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GLIAL MITOGENIC FACTORS, THEIR PREPARATION AND USE

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Cross Reference to Related Application

This application is a continuation-in-part of Serial No. 07/965,173, filed October 23, 1992, Serial No. 07/940,389, filed September 3, 1992, Serial No. 07/907,138, filed June 30, 1992 and Serial No. 07/863,703, filed April 3, 1992.

Background of the Invention

This invention relates to polypeptides found in vertebrate species, which polypeptides are mitogenic growth factors for glial cells, including Schwann cells. The invention is also concerned with processes capable of producing such factors, and the therapeutic application of such factors.

The glial cells of vertebrates constitute the specialized connective tissue of the central and peripheral nervous systems. Important glial cells include Schwann cells which provide metabolic support for neurons and which provide myelin sheathing around the axons of certain peripheral neurons, thereby forming individual nerve fibers. Schwann cells support neurons and provide a sheath effect by forming concentric layers of membrane around adjacent neural axons, twisting as they develop around the axons. These myelin sheaths are a susceptible element of many nerve fibers, and damage to Schwann cells, or failure in growth and development, can be associated with significant demyelination or nerve degeneration characteristic of a number of peripheral nervous system diseases and disorders. In the development of the nervous system, it has become apparent that cells require various factors to regulate their division and growth, and various such factors have been identified in recent years, including some found to have an effect on Schwann cell division or development.

Thus, Brockes et al., int r alia, in J. Neurosci nce, 4 (1984) 75-83 describ a prot in growth factor present in extracts from bovine brain and pituitary tissu , which was named Glial Growth Factor (GGF). This 5 factor stimulated cultured rat Schwann cells to divide against a background medium containing ten percent fetal calf serum. The factor was also described as having a molecular weight of 31,000 Daltons and as readily dimerizing. In Meth. Enz., 147 (1987), 217-225, Brockes 10 describes a Schwann cell-based assay for GGF.

Brockes et al., supra, also describes a method of purification of GGF to apparent homogeneity. In brief, one large-scale purification method described involves extraction of the lyophilized bovine anterior lobes and 15 chromatography of material obtained thereby using NaCl gradient elution from CM cellulose. Gel filtration is then carried out with an Ultrogel column, followed by elution from a phosphocellulose column, and finally, small-scale SDS gel electrophoresis. Alternatively, the CM-cellulose 20 material was applied directly to a phosphocellulose column, fractions from the column were pooled and purified by preparative native gel electrophoresis, followed by a final SDS gel electrophoresis.

Brockes et al. observe that in previously reported 25 gel filtration experiments (Brockes et al., J. Biol. Chem. 255 (1980) 8374-8377), the major peak of growth factor activity was observed to migrate with a molecular weight of 56,000 Daltons, whereas in the first of the above-described procedures activity was predominantly observed at molecular 30 weight 31,000. It is reported that the GGF dimer is largely removed as a result of the gradient elution from CM-cellulose in this procedure.

B nv nist t al. (PNAS, 82 (1985), 3930-3934) describe a T lymphocyt -derived glial growth pr moting factor. This factor, under reducing onditions, exhibits a change in apparent mol cular weight n SDS gels.

5 Kimura et al. (Nature, 348 (1990), 257-260) describe a factor they term Schwannoma-derived growth factor (SDGF) which is obtained from a sciatic nerve sheath tumor. The authors state that SDGF does not stimulate the incorporation of tritium-labelled Tdr into cultured Schwann cells under 10 conditions where, in contrast, partially purified pituitary fraction containing GGF is active. SDGF has an apparent molecular weight of between 31,000 and 35,000.

15 Davis and Stroobant (J. Cell. Biol., 110 (1990), 1353-1360) describe the screening of a number of candidate mitogens. Rat Schwann cells were used, the chosen candidate substances being examined for their ability to stimulate DNA synthesis in the Schwann cells in the presence of 10% FCS (fetal calf serum), with and without forskolin. One of the factors tested was GGF-carboxymethyl cellulose fraction 20 (GGF-CM), which was mitogenic in the presence of FCS, with and without forskolin. The work revealed that in the presence of forskolin, inter alia, platelet derived growth factor (PDGF) was a potent mitogen for Schwann cells, PDGF having previously been thought to have no effect on Schwann 25 cells.

Holmes et al. Science (1992) 256: 1205 and Wen et al. Cell (1992) 69: 559 demonstrate that DNA sequences which encode proteins binding to a receptor ($p185^{erbB2}$) are associated with several human tumors.

30 The $p185^{erbB2}$ protein is a 185 kilodalton membrane spanning protein with tyrosine kinase activity. The protein is encoded by the erbB2 proto-oncogene (Yarden and Ullrich Ann. Rev. Biochem. 57: 443 (1988)). The erbB2 gene, also

referred to as HER-2 (in human 11s) and neu (in rat cells), is closely related to the receptor for epidermal growth factor (EGF). Recent evidence indicates that proteins which interact with (and activate the kinase of) p185^{erbB2} induce proliferation in the cells bearing p185^{erbB2} (Holmes et al. *Science* **256**: 1205 (1992); Dobashi et al. Proc. Natl. Acad. Sci. **88**: 8582 (1991); Lupu et al. Proc. Natl. Acad. Sci. **89**: 2287 (1992)). Furthermore, it is evident that the gene encoding p185^{erbB2} binding proteins produces a number of variably-sized, differentially-spliced RNA transcripts that give rise to a series of proteins, which are of different lengths and contain some common peptide sequences and some unique peptide sequences. This is supported by the differentially-spliced RNA transcripts recoverable from human breast cancer (MDA-MB-231) (Holmes et al. *Science* **256**: 1205 (1992)). Further support derives from the wide size range of proteins which act as (as disclosed herein) ligands for the p185^{erbB2} receptor (see below).

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Summary of the Invention

In general the invention provides methods for stimulating glial cell (in particular, Schwann cell and glia of the central nervous system) mitogenesis, as well as new proteins exhibiting such glial cell mitogenic activity. In addition, DNA encoding these proteins and antibodies which bind these and related proteins are provided.

The novel proteins of the invention include alternative splicing products of sequences encoding known polypeptides. Generally, these known proteins are members of the GGF/p185^{erbB2} family of proteins.

Specifically, the invention provides polypeptides of a specified formula, and DNA sequences encoding those polypeptides. The polypeptides have the formula

WYBAZCX

- 5 wherein WYBAZCX is composed of the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136-139, 141-147, ✓ 160, 161); wherein W comprises the polypeptide segment F, or is absent; wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G or 10 is absent; and wherein X comprises the polypeptide segments C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' D' HKL; 15 provided that, either
a) at least one of F, Y, B, A, Z, C, or X is of bovine origin; or
b) Y comprises the polypeptide segment E; or
c) X comprises the polypeptide segments C/D HKL, C/D 20 D, C/D' HKL, C/D C/D' HKL, C/D C/D' D, C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, C/D C/D' D' HKL, C/D' H, C/D C/D' H, or C/D C/D' HL.
In addition, the invention includes the DNA sequence comprising coding segments 5'FBA^{3'} as well as the with 25 corresponding polypeptide segments having the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136, 138, 139); the DNA sequence comprising the coding segments 5'FBA^{3'} as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 31 (SEQ ID 30 Nos. 136, 138, 140); the DNA sequence comprising the coding segments 5'FEBA^{3'} as well as the corresponding polypeptide segments

having the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136-139);

the DNA sequence comprising the coding segments 5'FEBA'3' as well as the corresponding polypeptide segments having the amino acid sequences shown in Figure 31 (SEQ ID Nos. 136-138, 140); and

the DNA sequence comprising the polypeptide coding segments of the GGF2HBSS cDNA clone (ATCC Deposit No. 75298, deposited September 2, 1992).

The invention further includes peptides of the formula FBA, FEBA, FBA' FEBA' and DNA sequences encoding these peptides wherein the polypeptide segments correspond to amino acid sequences shown in Figure 31, SEQ ID Nos. (136, 138 and 139), (136-139) and (136, 138 and 140) and (136-138 and 140) respectively. The purified GGF-II polypeptide (SEQ ID No. 167) is also included as a part of the invention.

Further included as an aspect of the invention are peptides and DNA encoding such peptides which are useful for the treatment of glia and in particular oligodendrocytes, microglia and astrocytes, of the central nervous system and methods for the administration of these peptides.

The invention further includes vectors including DNA sequences which encode the amino acid sequences, as defined above. Also included are a host cell containing the isolated DNA encoding the amino acid sequences, as defined above. The invention further includes those compounds which bind the p185^{erbB2} receptor and stimulate glial cell mitogenesis in vivo and/or in vitro.

Also a part of the invention are antibodies to the novel peptides described herein. In addition, antibodies to any of the peptides described herein may be used for the purification of polypeptides described herein. The

antibodies to the polypeptides may also be used for the therapeutic inhibition of glial cell mitogenesis.

The invention further provides a method for stimulating glial cell mitogenesis comprising contacting 5 glial cells with a polypeptide defined by the formula

WYBAZCX

wherein WYBAZCX is composed of the polypeptide segments shown in Figure 31 (SEQ ID Nos. 136-139, 141-147, 10 160, 161); wherein W comprises the polypeptide segment F, or is absent wherein Y comprises the polypeptide segment E, or is absent; wherein Z comprises the polypeptide segment G or is absent; and wherein X comprises the polypeptide segment C/D HKL, C/D H, C/D HL, C/D D, C/D' HL, C/D' HKL, C/D' H, C/D' D, C/D C/D' HKL, C/D C/D' H, C/D C/D' HL, C/D C/D' D, 15 C/D D' H, C/D D' HL, C/D D' HKL, C/D' D' H, C/D' D' HL, C/D' D' HKL, C/D C/D' D' H, C/D C/D' D' HL, or C/D C/D' D' HKL.

The invention also includes a method for the preparation of a glial cell mitogenic factor which consists of culturing modified host cells as defined above under 20 conditions permitting expression of the DNA sequences of the invention.

The peptides of the invention can be used to make a pharmaceutical or veterinary formulation for pharmaceutical or veterinary use. Optionally, the formulation may be used 25 together with an acceptable diluent, carrier or excipient and/or in unit dosage form.

A method for stimulating mitogenesis of a glial cell by contacting the glial cell with a polypeptide defined above as a glial cell mitogen in vivo or in vitro is also an 30 aspect of the invention. A method for producing a glial cell mitogenic effect in a vertebrate (preferably a mammal, more preferably a human) by administering an effective

amount of a polypeptide as defined is also a component of the invention.

Methods for treatment of diseases and disorders using the polypeptides described are also a part of the invention. For instance, a method of treatment or prophylaxis for a nervous disease or disorder can be effected with the polypeptides described. Also included are a method for the prophylaxis or treatment of a pathophysiological condition of the nervous system in which a cell type is involved which is sensitive or responsive to a polypeptide as defined are a part of the invention.

Included in the invention as well, are methods for treatment when the condition involves peripheral nerve damage; nerve damage in the central nervous system; neurodegenerative disorders; demyelination in peripheral or central nervous system; or damage or loss of Schwann cells oligodendrocytes, microglia, or astrocytes. For example a neuropathy of sensory or motor nerve fibers; or the treatment of a neurodegenerative disorder are included. In any of these cases, treatment consists of administering an effective amount of the polypeptide.

The invention also includes a method for inducing neural regeneration and/or repair by administering an effective amount of a polypeptide as defined above. Such a medicament is made by administering the polypeptide with a pharmaceutically effective carrier.

The invention includes the use of a polypeptide as defined above in the manufacture of a medicament.

The invention further includes the use of a polypeptide as defined above

-to immunize a mammal for producing antibodies, which can optionally be used for therapeutic or diagnostic purposes

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-in a competitive assay to identify or quantify molecules having receptor binding characteristics corresponding to those of the polypeptide; and/or

5 -for contacting a sample with a polypeptide, as mentioned above, along with a receptor capable of binding specifically to the polypeptide for the purpose of detecting competitive inhibition of binding to the polypeptide.

10 -in an affinity isolation process, optionally affinity chromatography, for the separation of a corresponding receptor.

15 The invention also includes a method for the prophylaxis or treatment of a glial tumor. This method consists of administering an effective amount of a substance which inhibits the binding of a factor as defined by the peptides above.

Furthermore, the invention includes a method of stimulating glial cell mitogenic activity by the application to the glial cell of a

20 -30 kD polypeptide factor isolated from the MDA - MB 231 human breast cell line; or

-35 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line to the glial cell or

-75 kD polypeptide factor isolated from the SKBR-3 human breast cell line; or

25 -44 kD polypeptide factor isolated from the rat I-EJ transformed fibroblast cell line; or

-25kD polypeptide factor isolated from activated mouse peritoneal macrophages; or

30 -45 kD polypeptide factor isolated from the MDA - MB 231 human breast cell; or

-7 to 14 kD polypeptide factor isolated from the ATL-2 human T-cell line to the glial cell; or

-25 kD polypeptide factor isolated from the bovine kidney cells; and

-42 kD polypeptide factor (ARIA) isolated from brains.

5 The invention further includes a method for the use of the EGFL1, EGFL2, EGFL3, EGFL4, EGFL5, and EGFL6 polypeptides, Figure 38 to 43 and SEQ ID Nos. 154 to 159, respectively, for the stimulation of glial cell mitogenesis in vivo and in vitro.

10 Also included in the invention is the administration of the GGF-II polypeptide whose sequence is shown in Figure 45 for the stimulation of glial cell mitogenesis.

15 An additional aspect of the invention includes the use of the above-referenced peptides for the purpose of stimulating Schwann cells to produce growth factors which may, in turn, be harvested for scientific or therapeutic use.

20 Furthermore, the peptides described herein may be used to induce central glial proliferation and remyelination for treatment of diseases, e.g., MS, where re-myelination is desired.

25 In an additional aspect of the invention, the novel polypeptides described herein may be used to stimulate the synthesis of acetylcholine receptors.

As mentioned above, the invention provides new glial growth factors from mammalian sources, including bovine and human, which are distinguished from known factors. These factors are mitogenic for Schwann cells against a background of fetal calf plasma (FCP). The invention also provides processes for the preparation of these factors, and an improved method for defining activity of these and other factors. Therapeutic application of the factors is a further significant aspect of the invention.

Thus, important aspects of the invention are:

- (a) a basic polypeptide factor having glial cell mitogenic activity, more specifically, Schwann cell mitogenic activity in the presence of fetal calf plasma, a molecular weight of from about 30 kD to about 36 kD, and including within its amino acid sequence any one or more of the following peptide sequences:

(SEQ ID NO: 1)

C F K G D A H T E (SEQ ID NO: 2)
C A S L A D E Y E Y M X K (SEQ ID NO: 22)
100 T E T S S S G L X L K (SEQ ID NO: 23)
C A S L A D E Y E Y M R K (SEQ ID NO: 24)
C A G Y F A E X A R (SEQ ID NO: 25)
C T T E M A S E Q G A (SEQ ID NO: 26)
C A K E A L A A L K (SEQ ID NO: 27)
25 F V L Q A K K (SEQ ID NO: 28)
C E T Q P D P G Q I L K K V P M V I G A Y T (SEQ ID NO: 29)
C E Y K C L K F K W F K K A T V M (SEQ ID NO: 30)
C E X K F Y V P (SEQ ID NO: 31)
C K L E F L X A K; and (SEQ ID NO: 32)

- 20 (b) a basic polypeptide factor which stimulates glial cell mitogenesis, particularly the division of Schwann cells, in the presence of fetal calf plasma, has a molecular weight of from about 55 kD to about 63 kD, and including within its amino acid sequence any one or more of the
25 following peptide sequences:

(SEQ ID NO: 45)

C V H Q V W A A K (SEQ ID NO: 46)
C Y I F F M E P E A X S S G (SEQ ID NO: 47)
C L G A W G P P A F P V X Y (SEQ ID NO: 48)
C W F V V I E G K (SEQ ID NO: 49)
30 A S P V S V G S V Q E L Q R (SEQ ID NO: 50)

(SEQ ID NO:50)

V C L L T V A A L P P T
C K V H Q V W A A K ~ (SEQ ID NO:51)
C K A S L A D S G E Y M X K ~ (SEQ ID NO:52)
C D L L L X V ~ (SEQ ID NO:53)
5. C E G K V H P Q R R G A L D R K ~ (SEQ ID NO:185)
C P S C G R L K E D S R Y I F F M E ~ (SEQ ID NO:186)
C E L N R K N K P Q N I K I Q K K ~ (SEQ ID NO: 187)

The novel peptide sequences set out above, derived from the smaller molecular weight polypeptide factor, and 10 from the larger molecular weight polypeptide factor, are also aspects of this invention in their own right. These sequences are useful as probe sources for polypeptide factors of the invention, for investigating, isolating or preparing such factors (or corresponding gene sequences) 15 from a range of different species, or preparing such factors by recombinant technology, and in the generation of corresponding antibodies, by conventional technologies, preferably monoclonal antibodies, which are themselves useful investigative tools and are possible therapeutics.

20 The invention also includes an isolated glial cell mitogenic activity encoding gene sequence, or fragment thereof, obtainable by the methods set out above for the novel peptide sequences of the invention.

The availability of short peptides from the highly purified factors of the invention has enabled additional sequences to be determined (see Examples to follow).

Thus, the invention further embraces a polypeptide factor having glial cell mitogenic activity and including an amino acid sequence encoded by:

30 (a) a DNA sequence shown in any one of Figures 28a, 28b or 28c, SEQ ID Nos. 133-135, respectively;

- (b) a DNA sequence shown in Figure 22, SEQ ID No. 89;
- (c) the DNA sequence represented by nucleotides 281-557 of the sequence shown in Figure 28a, SEQ ID No. 133;
- 5 or
- (d) a DNA sequence hybridizable to any one of the DNA sequences according to (a), (b) or (c).

The invention further includes sequences which have greater than 60%, preferably 80%, sequence identity of
10 homology to the sequences indicated above.

While the present invention is not limited to a particular set of hybridization conditions, the following protocol gives general guidance which may, if desired, be followed:

15 DNA probes may be labelled to high specific activity (approximately 10^8 to 10^9 ^{32}P dNTP/ μg) by nick-translation or by PCR reactions according to Schowalter and Sommer (Anal. Biochem., 177:90-94, 1989) and purified by desalting on G-150 Sephadex columns. Probes may be denatured (10 minutes
20 in boiling water followed by immersion into ice water), then added to hybridization solutions of 80% buffer B (2g polyvinylpyrrolidine, 2g Ficoll-400, 2g bovine serum albumin, 50ml 1 M Tris HCl (pH 7.5), 58g NaCl, 1g sodium pyrophosphate, 10g sodium dodecyl sulfate, 950ml H_2O)
25 containing 10% dextran sulfate at 10^6 dpm ^{32}P per ml and incubated overnight (approximately 16 hours) at 60°C. The filters may then be washed at 60°C, first in buffer B for 15 minutes followed by three 20-minute washes in 2X SSC, 0.1% SDS then one for 20 minutes in 1x SSC, 0.1% SDS.

30 In other respects, the invention provides:

(a) a basic polypeptide factor which has, if obtained from bovine pituitary material, an observed molecular weight, whether in reducing conditions or not, of

from about 30kD to about 36kD on SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

5	Lysozyme (hen egg white)	14,400
	Soybean trypsin inhibitor	21,500
	Carbonic anhydrase (bovine)	31,000
	Ovalbumin (hen egg white)	45,000
	Bovine serum albumin	66,200
	Phosphorylase B (rabbit muscle)	97,400;

- 10 which factor has glial cell mitogenic activity including stimulating the division of rat Schwann cells in the presence of fetal calf plasma, and when isolated using reversed-phase HPLC retains at least 50% of said activity after 10 weeks incubation in 0.1% trifluoroacetic acid at
15 4°C; and

(b) a basic polypeptide factor which has, if obtained from bovine pituitary material, an observed molecular weight, under non-reducing conditions, of from about 55 kD to about 63 kD on SDS-polyacrylamide gel

- 20 electrophoresis using the following molecular weight standards:

25	Lysozyme (hen egg white)	14,400
	Soybean trypsin inhibitor	21,500
	Carbonic anhydrase (bovine)	31,000
	Ovalbumin (hen egg white)	45,000
	Bovine serum albumin	66,200
	Phosphorylase B (rabbit muscle)	97,400;

- which factor the human equivalent of which is encoded by DNA clone GGF2HBS5 described herein and which factor has glial cell mitogenic activity including stimulating the division
30

of rat Schwann cells in the presence of fetal calf plasma, and when isolated using reversed-phase HPLC retains at least 50% of the activity after 4 days incubation in 0.1% trifluoroacetic acid at 4°C.

5 For convenience of description only, the lower molecular weight and higher molecular weight factors of this invention are referred to hereafter as "GGF-I" and "GGF-II", respectively. The "GGF2" designation is used for all clones isolated with peptide sequence data derived from GGF-II protein (i.e., GGF2HBS5, GGF2BPP3).

10 It will be appreciated that the molecular weight range limits quoted are not exact, but are subject to slight variations depending upon the source of the particular polypeptide factor. A variation of, say, about 10% would 15 not, for example, be impossible for material from another source.

Another important aspect of the invention is a DNA sequence encoding a polypeptide having glial cell mitogenic activity and comprising:

- 20 (a) a DNA sequence shown in any one of Figures 28a, 28b or 28c, SEQ ID Nos. 133-135;
- 25 (b) a DNA sequence shown in Figure 22, SEQ ID No. 89;
- (c) the DNA sequence represented by nucleotides 281-557 of the sequence shown in Figure 28a, SEQ ID No. 133; or
- (d) a DNA sequence hybridizable to any one of the DNA sequences according to (a), (b) or (c).

30 Another aspect of the present invention uses the fact that the Glial Growth Factors and p185^{erbB2} ligand proteins are encoded by the same gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these

products show p185^{erbB2} binding and activation. Several of the (GGF-II) gene products have been used to show Schwann cell mitogenic activity. This invention provides a use for all of the known products of the GGF/p185^{erbB2} ligand gene (described in the references listed above) as Schwann cell mitogens.

This invention also relates to other, not yet naturally isolated splicing variants of the Glial Growth Factor gene. Figure 30, shows the known patterns of splicing derived from polymerase chain reaction experiments (on reverse transcribed RNA) and analysis of cDNA clones (as presented within) and derived from what has been published as sequences encoding p185^{erbB2} ligands (Peles et al., Cell 69:205 (1992) and Wen et al., Cell 69:559 (1992)). These patterns, as well as additional ones disclosed herein, represent probable splicing variants which exist. Thus another aspect of the present invention relates to the nucleotide sequences encoding novel protein factors derived from this gene. The invention also provides processes for the preparation of these factors. Therapeutic application of these new factors is a further aspect of the invention.

Thus other important aspects of the invention are :

(a) A series of human and bovine polypeptide factors having glial cell mitogenic activity including stimulating the division of Schwann cells. These peptide sequences are shown in Figures 31, 32, 33 and 34, SEQ ID Nos. 136-137, respectively.

(b) A series of polypeptide factors having glial cell mitogenic activity including stimulating the division of Schwann cells and purified and characterized according to the procedures outlined by Lupu et al. Science 249: 1552 (1990); Lupu et al. Proc. Natl. Acad. Sci USA 89: 2287 (1992); Holmes et al. Science 256: 1205 (1992); Peles et al.

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69: 205 (1992); Yard n and P l s Biochemistry 30: 3543
1991); Dobashi t al. Proc. Natl. Acad. Sci. 88: 8582
1991); Davis et al. Biochem. Biophys. Res. Commun. 179:
1536 (1991); Beaumont et al., patent application
5 PCT/US91/03443 (1990); Greene et al. patent application
PCT/US91/02331 (1990); Usdin and Fischbach, J. Cell. Biol.
103:493-507 (1986); Falls et al., Cold Spring Harbor Symp.
Quant. Biol. 55:397-406 (1990); Harris et al., Proc. Natl.
Acad. Sci. USA 88:7664-7668 (1991); and Falls et al., Cell
10 72:801-815 (1993).

(c) A polypeptide factor (GGFBPP5) having glial cell mitogenic activity including stimulating the division of Schwann cells. The amino acid sequence is shown in Figure 32, SEQ ID No. 148, and is encoded by the bovine DNA sequence shown in Figure 32, SEQ ID No. 148.

The novel human peptide sequences described above and presented in Figures 31, 32, 33 and 34, SEQ ID Nos. 136-150, respectively, represent a series of splicing variants which can be isolated as full length complementary DNAs (cDNAs) from natural sources (cDNA libraries prepared from the appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the art.

Other compounds in particular, peptides, which bind specifically to the p185^{erbB2} receptor can also be used according to the invention as a glial cell mitogen. A candidate compound can be routinely screened for p185^{erbB2} binding, and, if it binds, can then be screened for glial cell mitogenic activity using the methods described herein.

30 The invention includes any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity. For example, modifications in which amino acid content or sequence is

altered without substantially adversely affecting activity
are included. By way of illustration, in EP-A 109748
mutations of native proteins are disclosed in which the
possibility of unwanted disulfide bonding is avoided by
5 replacing any cysteine in the native sequence which is not
necessary for biological activity with a neutral amino acid.
The statements of effect and use contained herein are
therefore to be construed accordingly, with such uses and
effects employing modified or equivalent factors being part
10 of the invention.

The new sequences of the invention open up the
benefits of recombinant technology. The invention thus also
includes the following aspects:

(a) DNA constructs comprising DNA sequences as
15 defined above in operable reading frame position within
vectors (positioned relative to control sequences so as to
permit expression of the sequences) in chosen host cells
after transformation thereof by the constructs (preferably
the control sequence includes regulatable promoters, e.g.
20 Trp). It will be appreciated that the selection of a
promoter and regulatory sequences (if any) are matters of
choice for those of skill in the art;

(b) host cells modified by incorporating constructs
as defined in (a) immediately above so that said DNA
25 sequences may be expressed in said host cells - the choice
of host is not critical, and chosen cells may be prokaryotic
or eukaryotic and may be genetically modified to incorporate
said constructs by methods known in the art; and,

(c) a process for the preparation of factors as
30 defined above comprising cultivating the modified host cells
under conditions permitting expression of the DNA sequences.
These conditions can be readily determined, for any
particular embodiment, by those of skill in the art of

recombinant DNA technology. Glial cell mitogens present by this means are included in the present invention.

None of the factors described in the art has the combination of characteristics possessed by the present new polypeptide factors.

As indicated, the Schwann cell assay used to characterize the present factors employs a background of fetal calf plasma. In all other respects, the assay can be the same as that described by Brockes et al. in Meth. Enz., supra, but with 10% FCP replacing 10% FCS. This difference in assay techniques is significant, since the absence of platelet-derived factors in fetal calf plasma (as opposed to serum) enables a more rigorous definition of activity on Schwann cells by eliminating potentially spurious effects from some other factors.

The invention also includes a process for the preparation of a polypeptide as defined above, extracting vertebrate brain material to obtain protein, subjecting the resulting extract to chromatographic purification by hydroxylapatite HPLC and then subjecting these fractions to SDS-polyacrylamide gel electrophoresis. The fraction which has an observed molecular weight of about 30kD to 36 kD and/or the fraction which has an observed molecular weight of about 55kD to 63 kD is collected. In either case, the fraction is subjected to SDS-polyacrylamide gel electrophoresis using the following molecular weight standards:

Lysozyme (hen egg white)	14,400
Soybean trypsin inhibitor	21,500
Carbonic anhydrase (bovine)	31,000
Ovalbumin (hen egg white)	45,000
Bovine serum albumin	66,200
Phosphorylase B (rabbit muscle)	97,400

In th case of th small r molecular weight fraction, th SDS-polyacrylamid gel is run in non-r ducing conditions in r ducing conditi ns r, and in th cas of the larg r molecular weight fracti n th gel is run und r non-r ducing 5 conditions. The fractions are then tested for activity stimulating the division of rat Schwann cells against a background of fetal calf plasma.

Preferably, the above process starts by isolating a relevant fraction obtained by carboxymethyl cellulose chromatography, e.g. from bovine pituitary material. It is also preferred that hydroxylapatite HPLC, cation exchange chromatography, gel filtration, and/or reversed-phase HPLC be employed prior to the SDS-Polyacrylamide gel electrophoresis. At each stage in the process, activity may 10 be determined using Schwann cell incorporation of radioactive iododeoxyuridine as a measure in an assay generally as described by Brockes in Meth. Enz., supra, but modified by substituting 10% FCP for 10% FCS. As already noted, such as assay is an aspect of the invention in its 15 own substance for CNS or PNS cell, e.g. Schwann cell, mitogenic effects.

Thus, the invention also includes an assay for glial cell mitogenic activity in which a background of fetal calf plasma is employed against which to assess DNA synthesis in 20 glial cells stimulated (if at all) by a substance under assay.

Another aspect of the invention is a pharmaceutical or veterinary formulation comprising any factor as defined above formulated for pharmaceutical or veterinary use, 25 respectively, optionally together with an acceptable diluent, carrier or excipient and/or in unit dosage form. In using the factors of the invention, conventional

pharmaceutical or veterinary practice may be employed to provide suitable formulations or compositions.

Thus, the formulations of this invention can be applied to parenteral administration, for example, 5 intravenous, subcutaneous, intramuscular, intraorbital, ophthalmic, intraventricular, intracranial, intracapsular, intraspinal, intracisternal, intraperitoneal, topical, intranasal, aerosol, scarification, and also oral, buccal, rectal or vaginal administration.

10 The formulations of this invention may also be administered by the transplantation into the patient of host cells expressing the DNA of the instant invention or by the use of surgical implants which release the formulations of the invention.

15 Parenteral formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

20 Methods well known in the art for making formulations are to be found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain as excipients sterile water or saline, polyalkylene glycols such as 25 polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes, biocompatible, biodegradable lactide polymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the present factors. Other potentially useful parenteral delivery 30 systems for the factors include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain as excipients, for example, lactose, or may be

aqueous solutions containing, for example,
polyoxyethylen -9-lauryl ether, glycocholate and
deoxycholate, may be oily solutions for administration in
the form of nasal drops, or as a gel be applied
intranasally. Formulations for parenteral administration
may also include glycocholate for buccal administration,
methoxysalicylate for rectal administration, or citric acid
for vaginal administration.

The present factors can be used as the sole active
agents, or can be used in combination with other active
ingredients, e.g., other growth factors which could
facilitate neuronal survival in neurological diseases, or
peptidase or protease inhibitors.

The concentration of the present factors in the
formulations of the invention will vary depending upon a
number of issues, including the dosage to be administered,
and the route of administration.

In general terms, the factors of this invention may
be provided in an aqueous physiological buffer solution
containing about 0.1 to 10% w/v compound for parenteral
administration. General dose ranges are from about 1 mg/kg
to about 1 g/kg of body weight per day; a preferred dose
range is from about 0.01 mg/kg to 100 mg/kg of body weight
per day. The preferred dosage to be administered is likely
to depend upon the type and extent of progression of the
pathophysiological condition being addressed, the overall
health of the patient, the make up of the formulation, and
the route of administration.

As indicated above, Schwann cells (the glial cells
of the peripheral nervous system) are stimulated to divide
in the presence of the factors of the invention. Schwann
cells of the peripheral nervous system are involved in
supporting neurons and in creating the myelin sheath around

individual nerv fibers. This sheath is important f r proper conduction f el ctrical impulses to muscl s and from sensory receptors.

There are a variety of peripheral neuropathies in
5 which Schwann cells and nerve fibers are damaged, either primarily or secondarily. There are many neuropathies of both sensory and motor fibers (Adams and Victor, Principles of Neurology). The most important of those neuropathies are probably the neuropathies associates with diabetes, multiple
10 sclerosis, Landry-Guillain-Barr syndrome, neuropathies caused by carcinomas, and neuropathies caused by toxic agents (some of which are used to treat carcinomas).

The invention, however, envisages treatment or prophylaxis of conditions where nervous system damage has
15 been brought about by any basic cause, e.g. infection or injury. Thus, in addition to use of the present factors in the treatment of disorders or diseases of the nervous system where demyelination or loss of Schwann cells is present, such glial growth factors can be valuable in the treatment
20 of disorders of the nervous system that have been caused by damage to the peripheral nerves. Following damage to peripheral nerves, the regeneration process is led by the growth or the re-establishment of Schwann cells, followed by the advancement of the nerve fibre back to its target. By
25 speeding up the division of Schwann cells one could promote the regenerative process following damage.

Similar approaches could be used to treat injuries or neurodegenerative disease of the central nervous system (brain and spinal cord).

30 Furthermore, there are a variety of tumors of glial cells the most common of which is probably neurofibromatosis, which is a patchy small tumor created by overgrowth of glial cells. Also, it has been found that an

activity very much like GGF can be found in some Schwann cell tumors, and therefore inhibitors of the action of the present factors on their receptors provides a therapy of a glial tumor, which comprises administering an effective

5 amount of a substance which inhibits the binding of a factor, as defined above, to a receptor.

In general, the invention includes the use of present polypeptide factors in the prophylaxis or treatment of any pathophysiological condition of the nervous system in
10 which a factor-sensitive or factor-responsive cell type is involved.

The polypeptide factors of the invention can also be used as immunogens for making antibodies, such as monoclonal antibodies, following standard techniques. Such antibodies
15 are included within the present invention. These antibodies can, in turn, be used for therapeutic or diagnostic purposes. Thus, conditions perhaps associated with abnormal levels of the factor may be tracked by using such antibodies. In vitro techniques can be used, employing
20 assays on isolated samples using standard methods. Imaging methods in which the antibodies are, for example, tagged with radioactive isotopes which can be imaged outside the body using techniques for the art of tumour imaging may also be employed.

25 The invention also includes the general use of the present factors as glial cell mitogens in vivo or in vitro, and the factors for such use. One specific embodiment is thus a method for producing a glial cell mitogenic effect in a vertebrate by administering an effective amount of a
30 factor of the invention. A preferred embodiment is such a method in the treatment or prophylaxis of a nervous system disease or disorder.

A further general aspect of the invention is the use of a factor of the invention in the manufacture of a medicament, preferably for the treatment of a nervous disease or disorder, or for neural regeneration or repair.

5 Also included in the invention are the use of the factors of the invention in competitive assays to identify or quantify molecules having receptor binding characteristics corresponding to those of said polypeptides. The polypeptides may be labelled, optionally with a
10 radioisotope. A competitive assay can identify both antagonists and agonists of the relevant receptor.

In another aspect, the invention provides the use of each one of the factors of the invention in an affinity isolation process, optionally affinity chromatography, for
15 the separation of a respective corresponding receptor. Such processes for the isolation of receptors corresponding to particular proteins are known in the art, and a number of techniques are available and can be applied to the factors of the present invention. For example, in relation to IL-6 and IFN γ the reader is referred to Novick, D.; et al., J. Chromatogr. (1990) 510: 331-7. With respect to gonadotropin releasing hormone reference is made to Hazum, E., J. (1990) Chromatogr. 510:233-8. In relation to G-CSF reference is made to Fukunaga, R., et al., J. Biol. Chem., 265:13386-90.
20 In relation to IL-2 reference is made to Smart, J.E., et al., (1990) J. Invest. Dermatol., 94:158S-163S, and in relation to human IFN-gamma reference is made to Stefanos, S, et al., (1989) J. Interferon Res., 9:719-30.
25

Brief Description of the Drawings

30 The drawings will first be described.

Drawings

DOVER EDITIONS
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Figures 1 to 8 relate to Example 1, and are briefly described below:

Fig. 1 is the profile for product from carboxymethyl cellulose chromatography;

5 Fig. 2 is the profile for product from hydroxylapatite HPLC;

Fig. 3 is the profile for product from Mono S FPLC;

Fig. 4 is the profile for product from Gel filtration FPLC;

10 Figs. 5 and 6 depict the profiles for the two partially purified polypeptide products from reversed-phase HPLC; and

15 Figs. 7 and 8 depict dose-response curves for the GGF-I and GGF-II fractions from reversed-phase HPLC using either a fetal calf serum or a fetal calf plasma background;

Figs. 9 to 12 depict the peptide sequences derived from GGF-I and GGF-II, SEQ ID Nos. 1-20, 22-29, 32-53 and 169, (see Example 2 hereinafter), Figures 10 and 12 specifically depict novel sequences:

In Fig. 10^(A-15), Panel A, the sequences of GGF-I peptides used to design degenerate oligonucleotide probes and degenerate PCR primers are listed (SEQ ID Nos. 20, 1, 22-29, and 17). Some of the sequences in Panel A were also used to design synthetic peptides. Panel B is a listing of the 25 sequences of novel peptides that were too short (less than 6 amino acids) for the design of degenerate probes or degenerate PCR primers (SEQ ID Nos. 17 and 52);

In Fig. 12^(A-B), Panel A, is a listing of the sequences of GGF-II peptides used to design degenerate oligonucleotide probes and degenerate PCR primers (SEQ ID Nos. 45-52). Some of the sequences in Panel A were used to design synthetic peptides. Panel B is a listing of the novel peptide that

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was too short (less than 6 amino acids) for the design of degenerate probes and generate PCR primers (SEQ ID No. 53);

Figures 13 to 20 relate to Example 3, below and depict the mitogenic activity of factors of the invention;

Figures 21 to 28 (a, b and c) relate to Example 4, below and are briefly described below:

Fig. 21 is a listing of the degenerate oligonucleotide probes (SEQ ID Nos. 54-88) designed from the novel peptide sequences in Figure 10, Panel A and Figure 12,

10 Panel A;

Fig. 22 (SEQ ID No. 89) depicts a stretch of the putative bovine GGF-II gene sequence from the recombinant bovine genomic phage GGF2BG1, containing the binding site of degenerate oligonucleotide probes 609 and 650 (see Figure 21, SEQ ID NOS. 69 and 72, respectively). The figure is the coding strand of the DNA sequence and the deduced amino acid sequence in the third reading frame. The sequence of peptide 12 from factor 2 (bold) is part of a 66 amino acid open reading frame (nucleotides 75272);

Fig. 23 is the degenerate PCR primers (Panel A, SEQ ID Nos. 90-108) and unique PCR primers (Panel B, SEQ ID Nos. 109-119) used in experiments to isolate segments of the bovine GGF-II coding sequences present in RNA from posterior pituitary;

Fig. 24 depicts of the nine distinct contiguous bovine GGF-II cDNA structures and sequences that were obtained in PCR amplification experiments using the list of primers in Figure 7, Panels A and B, and RNA from posterior pituitary. The top line of the Figure is a schematic of the coding sequences which contribute to the cDNA structures that were characterized;

Fig. 25 is a physical map of bovine recombinant phage of GGF2BG1. The bovine fragment is roughly 20 kb in

length and contains two exons (bold) of the bovine GGF-II gene. Restriction sites for the enzymes XbaI, SpeI, NdI, EcoRI, KpnI, and SstI have been placed on this physical map. Shaded portions correspond to fragments which were subcloned

5 for sequencing;

Fig. 26 is a schematic of the structure of three alternative gene products of the putative bovine GGF-II gene. Exons are listed A through E in the order of their discovery. The alternative splicing patterns 1, 2 and 3 generate three overlapping deduced protein structures (GGF2BPP1, 2, and 3), which are displayed in the various Figures 28a, b, c (described below);

Fig. 27 (SEQ ID Nos. 120-132) is a comparison of the GGF-I and GGF-II sequences identified in the deduced protein sequences shown in Figures 28a, 28b and 28c (described below) with the novel peptide sequences listed in Figures 10 and 12. The Figure shows that six of the nine novel GGF-II peptide sequences are accounted for in these deduced protein sequences. Two peptide sequences similar to GGF-I sequences are also found;

Fig. 28a (SEQ ID No. 133) is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern number 1 in Figure 26. This partial cDNA of the putative bovine GGF-II gene encodes a protein of 206 amino acids in length. Peptides in bold were those identified from the lists presented in Figures 10 and 12. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA);

Fig. 28b (SEQ ID No. 134) is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern number 2 in Figure 26. This partial cDNA of the putative bovine GGF-II gene encodes a protein of 281 amino acids in length. Peptides in bold

ar those identified from the lists presented in Figures 10 and 12. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA);

Fig. 28c (SEQ ID No. 135) is a listing of the coding strand DNA sequence and deduced amino acid sequence of the cDNA obtained from splicing pattern number 3 in Figure 26. This partial cDNA of the putative bovine GGF-II gene encodes a protein of 257 amino acids in length. Peptides in bold are those identified from the lists in Figures 10 and 12. Potential glycosylation sites are underlined (along with polyadenylation signal AATAAA).

Fig. 29, which relates to Example 6 hereinafter, is an autoradiogram of a cross hybridization analysis of putative bovine GGF-II gene sequences to a variety of mammalian DNAs on a southern blot. The filter contains lanes of EcoRI-digested DNA (5 µg per lane) from the species listed in the Figure. The probe detects a single strong band in each DNA sample, including a four kilobase fragment in the bovine DNA as anticipated by the physical map in Figure 25. Bands of relatively minor intensity are observed as well, which could represent related DNA sequences. The strong hybridizing band from each of the other mammalian DNA samples presumably represents the GGF-II homologue of those species.

Fig. 30 is a diagram of representative splicing variants. The coding segments are represented by F, E, B, A, G, C, C/D, C/D', D, D', H, K and L. The location of the peptide sequences derived from purified protein are indicated by "o".

Fig. 31 (SEQ ID Nos. 136-147, 160, 161) is a listing of the DNA sequences and predicted peptide sequences of the coding segments of GGF. Line 1 is a listing of the predicted amino acid sequences of bovine GGF, line 2 is a

listing of the nucleotide sequences of bovine GGF, line 3 is a listing of the nucleotide sequences of human GGF (heregulin) (nucleotide base matches are indicated with a vertical line) and line 4 is a listing of the predicted amino acid sequences of human GGF/heregulin where it differs from the predicted bovine sequence. Coding segments E, A' and K represent only the bovine sequences. Coding segment D' represents only the human (heregulin) sequence.

Fig. 32^{A and 32B} (SEQ ID No. 148) is the predicted GGF2 amino acid sequence and nucleotide sequence of BPP5. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

Fig. 33^{A and 33B} (SEQ ID No. 149) is the predicted amino acid sequence and nucleotide sequence of GGF2BPP2. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

Fig. 34^{A 34B and 34C} (SEQ ID No. 150) is the predicted amino acid sequence and nucleotide sequence of GGF2BPP4. The upper line is the nucleotide sequence and the lower line is the predicted amino acid sequence.

Fig. 35 (SEQ ID Nos. 151-152) depicts the alignment of two GGF peptide sequences (GGF2bpp4 and GGF2bpp5) with the human EGF (hEGF). Asterisks indicate positions of conserved cysteines.

Fig. 36 depicts the level of GGF activity (Schwann cell mitogenic assay) and tyrosine phosphorylation of a ca. 200kD protein (intensity of a 200 kD band on an autoradiogram of a Western blot developed with an antiphosphotyrosine polyclonal antibody) in response to increasing amounts of GGF.

Fig. 37^{37A and 37B} is a list of splicing variants derived from the sequences shown in Figure 31.

Fig. 38 is the predicted amino acid sequence,
bottom, and nucleic sequence, top, of EGFL1 (SEQ ID No.
154).

Fig. 39 is the predicted amino acid sequence,
5 bottom, and nucleic sequence, top, of EGFL2 (SEQ ID No.
155).

Fig. 40 is the predicted amino acid sequence,
bottom, and nucleic sequence, top, of EGFL3 (SEQ ID No.
156).

10 Fig. 41 is the predicted amino acid sequence,
bottom, and nucleic sequence, top, of EGFL4 (SEQ ID No.
157).

Fig. 42 is the predicted amino acid sequence,
bottom, and nucleic sequence, top, of EGFL5 (SEQ ID No.
15 158).

Fig. 43 is the predicted amino acid sequence,
bottom, and nucleic sequence, top, of EGFL6 (SEQ ID No.
159).

Fig. 44 is a scale coding segment map of the clone.
20 T3 refers to the bacteriophage promoter used to produce mRNA
from the clone. R = flanking EcoRI restriction enzyme
sites. 5' UT refers to the 5' untranslated region. E, B,
A, C, C/D', and D refer to the coding segments. O = the
translation start site. \wedge = the 5' limit of the region
25 homologous to the bovine E segment (see example 6) and 3' UT
refers to the 3' untranslated region.

C Fig. 45 is the predicted amino acid sequence
C ^{A 45B 45C and 45D} (middle) and nucleic sequence (top) of GGF2HBS5 (SEQ ID No.
^{170₂₁}₁₆₇) ¹⁷¹. The bottom (intermittent) sequence represents peptide
30 sequences derived from GGF-II preparations (see Figures 11,
12).

Fig. 46 is a graph depicting the Schwann cell mitogenic activity of recombinant human and bovine glial growth factors.

Fig. 47 is a dose-response curve depicting Schwann cell proliferation activity data resulting from administration of different size aliquots of CHO cell conditioned medium.

Fig. 48 is a dose-response curve depicting Schwann cell mitogenic activity secreted into the extracellular medium by SF9 insect cells infected with baculovirus containing the GGF2HBSS5 cDNA clone.

Fig. 49 is a Western blot of recombinant CHO cell conditioned medium using a GGF peptide antibody.

Fig. 50 (A) is a graph of Schwann cell proliferation activity of recombinant (COS cell produced) human GGF-II (rhGGF-II) peak eluted from the cation exchange column; (B) is an immunoblot against recombinant GGFII peak using polyclonal antibody made against specific peptide of rhGGFII;

Fig. 51 (A) is a graph showing the purification of rhGGF-II (CHO cell produced) on cation exchange column by fraction; (B) is a photograph of a Western blot using fractions as depicted in (A) and a rhGGF-II specific antibody.

Fig. 52 is a photograph of a gel depicting tyrosine phosphorylation in Schwann cells treated with recombinant glial growth factors.

Fig. 53 is the sequences of GGFHBS5, GGFHFB1 and GGFBBP5 polypeptides (SEQ ID NOS: 170, 171, and 172).

Fig. 54 is a map of the CHO cell-expression vector pcDHFpolyA.

Detailed Description

The invention pertains to the isolation and purification of novel Glial Growth factors and the cloning of DNA sequences encoding these factors. Other components of the invention are several gene splicing variants which potentially encode a series of glial growth factors, in particular the GGF2HBSS5 in particular a variant which encodes the human equivalent of bovine GGF-II. It is evident that the gene encoding GGF's and p185^{erbB2} binding proteins produces a number of variably-sized, differentially-spliced RNA transcripts that give rise to a series of proteins, which are of different lengths and contain some common peptide sequences and some unique peptide sequences. This is supported by the differentially-spliced sequences which are recoverable from bovine posterior pituitary RNA (as presented herein), human breast cancer (MDA-MB-231) (Holmes et al. *Science* **256**: 1205 (1992)) and chicken brain RNA (Falls et al. *Cell* **72**:1-20 (1993)). Further support derives from the wide size range of proteins which act as both mitogens for Schwann cells (as disclosed herein) and as ligands for the p185^{erbB2} receptor (see below).

Further evidence to support the fact that the genes encoding GGF and p185^{erbB2} are homologous comes from nucleotide sequence comparison. *Science*, **256** (1992), 1205-1210) Holmes et al. demonstrate the purification of a 45-kilodalton human protein (Heregulin- α) which specifically interacts with the receptor protein p185^{erbB2}, which is associated with several human malignancies. Several complementary DNA clones encoding Heregulin- α were isolated. Peles et al. (*Cell* **69**:205 (1992)) and Wen et al (*Cell* **69**:559 (1992)) describe a complementary DNA isolated from rat cells encoding a protein called "neu differentiation factor"

(NDF). The translation product of the NDF cDNA has p185^{erbB2} binding activity. Usdin and Fischbach, J. Cell. Biol. 103:493-507 (1986); Falls et al., Cold Spring Harbor Symp. Quant. Biol. 55:397-406 (1990); Harris et al., Proc. Natl. Acad. Sci. USA 88:7664-7668 (1991); and Falls et al., Cell 72:801-815 (1993) demonstrate the purification of a 42 Kd glycoprotein which interacts with a receptor protein p185^{erbB2} and several complementary cDNA clones were isolated (Falls et al. Cell 72:801-815 (1993)). Several other groups have reported the purification of proteins of various molecular weights with p185^{erbB2} binding activity. These groups include Lupu et al. (1992) Proc. Natl. Acad. Sci. USA 89:2287; Yarden and Peles (1991) Biochemistry 30:3543; Lupu et al. (1990) Science 249:1552); Dobashi et al. (1991) Biochem. Biophys. Res. Comm. 179:1536; and Huang et al. (1992) J. Biol. Chem. 257:11508-11512.

Other Embodiments

The invention includes any protein which is substantially homologous to the coding segments in Figure 31 (SEQ ID No.s 136-147, 160, and 161) as well as other naturally occurring GGF polypeptides. Also included are: allelic variations; natural mutants; induced mutants; proteins encoded by DNA that hybridizes under high or low stringency conditions to a nucleic acid naturally occurring (for definitions of high and low stringency see Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1989, 6.3.1 - 6.3.6, hereby incorporated by reference); and polypeptides or proteins specifically bound by antisera to GGF polypeptide. The term also includes chimeric polypeptides that include the GGF polypeptides comprising sequences from Figure 31.

The following examples are not intended to limit the invention, but are provided to usefully illustrate the same,

and provide specific guidance for effective preparative techniques.

- As will be seen from Example 3, below, the present factors exhibit mitogenic activity on a range of cell types.
- 5 The activity in relation to fibroblasts indicates a wound repair ability, and the invention encompasses this use. The general statements of invention above in relation to formulations and/or medicaments and their manufacture should clearly be construed to include appropriate products and
- 10 uses. This is clearly a reasonable expectation for the present invention, given reports of similar activities for fibroblast growth factors (FGFs). Reference can be made, for example, to Sporn *et al.*, "Peptide Growth Factors and their Receptors I", page 396 (Baird and Bohlen) in the
- 15 section headed "FGFs in Wound Healing and Tissue Repair".

EXAMPLE 1

Purification of GGF-I and GGF-II from bovine Pituitaries I. Preparation of Factor-CM Fraction

4,000 frozen whole bovine pituitaries (c.a. 12 kg) were thawed overnight, washed briefly with water and then homogenized in an equal volume of 0.15 M ammonium sulphate in batches in a Waring Blender. The homogenate was taken to pH 4.5 with 1.0 M HCl and centrifuged at 4,900g for 80 minutes. Any fatty material in the supernatant was removed by passing it through glass wool. After taking the pH of the supernatant to 6.5 using 1.0 M NaOH, solid ammonium sulphate was added to give a 36% saturated solution. After several hours stirring, the suspension was centrifuged at 4,900 g for 80 minutes and the precipitate discarded. After filtration through glass wool, further solid ammonium sulphate was added to the supernatant to give a 75% saturated solution which was once again centrifuged at 4,900 g for 80 minutes after several hours stirring. The pellet

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DISCUSSION

was resuspended in c.a. 2 L of 0.1 M sodium phosphate pH 6.0 and dialyzed against 3 x 40 L of the same buffer. After confirming that the conductivity of the dialysate was below 20.0 μ Siemens, it was loaded onto a Bioprocess column (120 x 5 113 mm, Pharmacia) packed with carboxymethyl cellulose (CM-52, Whatman) at a flow rate of 2 ml min⁻¹. The column was washed with 2 volumes of 0.1 M sodium phosphate pH 6.0, followed by 2 volumes of 50 mM NaCl, and finally 2 volumes of 0.2 M NaCl both in the same buffer. During the final 10 step, 10 mL (5 minute) fractions were collected. Fractions 73 to 118 inclusive were pooled, dialyzed against 10 volumes of 10 mM sodium phosphate pH 6.0 twice and clarified by centrifugation at 100,000 g for 60 minutes.

II. Hydroxylapatite HPLC

15 Hydroxylapatite HPLC is not a technique hitherto used in isolating glial growth factors, but proved particularly efficacious in this invention. The material obtained from the above CM-cellulose chromatography was filtered through a 0.22 μ m filter 20 (Nalgene), loaded at room temperature on to a high performance hydroxylapatite column (50 x 50 mm, Biorad) equipped with a guard column (15 x 25 mm, Biorad) and equilibrated with 10 mM potassium phosphate pH 6.0. Elution at room temperature was carried out at a flow rate of 2 25 ml.minute⁻¹ using the following programmed linear gradient:

time (min)	%B Solvent A:	10 mM potassium phosphate pH 6.0
0.0	0 Solvent B:	1.0 M potassium phosphate pH 6.0
5.0	0	
7.0	20	
30 70.0	20	
150.0	100	

180.0 100

185.0 0

6.0 mL (3 minutes) fractions were collected during the gradient elution. Fractions 39-45 were pooled and dialyzed against 10 volumes of 50 mM sodium phosphate pH 6.0.

III. Mono S FPLC

Mono S FPLC enabled a more concentrated material to be prepared for subsequent gel filtration.

Any particulate material in the pooled material from the hydroxylapatite column was removed by a clarifying spin at 100,000 g for 60 minutes prior to loading on to a preparative HR10/10 Mono S cation exchange column (100 x 10 mm, Pharmacia) which was then re-equilibrated to 50mM sodium phosphate pH 6.0 at room temperature with a flow rate of 1.0 ml/minute⁻¹. Under these conditions, bound protein was eluted using the following programmed linear gradient:

time (min)	%B	Solvent A: 50 mM potassium phosphate pH 6.0 Solvent B: 1.2 M sodium chloride, 50 mM sodium phosphate pH 6.0
0.0	0	
70.0	30	
20 240.0	100	
250.0	100	
260.0	0	

1 mL (1 minute) fractions were collected throughout this gradient program. Fractions 99 to 115 inclusive were pooled.

IV. Gel Filtration FPLC

This step commenced the separation of the two factors of the invention prior to final purification, producing enriched fractions.

For the purposes of this step, a preparative Superose 12 FPLC column (510 x 20 mm, Pharmacia) was packed according to the manufacturers' instructions. In order to standardize this column, a theoretical plates measurement 5 was made according to the manufacturers' instructions, giving a value of 9,700 theoretical plates.

The pool of Mono S eluted material was applied at room temperature in 2.5 mL aliquots to this column in 50 mM sodium phosphate, 0.75 M NaCl pH 6.0 (previously passed 10 through a C18 reversed phase column (Sep-pak, Millipore) at a flow rate of 1.0 mL/minute⁻¹. 1 mL (0.5 minute) fractions were collected from 35 minutes after each sample was applied to the column. Fractions 27 to 41 (GGF-II) and 42 to 57 (GGF-I) inclusive from each run were pooled.

15 V. Reversed-Phase HPLC

The GGF-I and GGF-II pools from the above Superose 12 runs were each divided into three equal aliquots. Each aliquot was loaded on to a C8 reversed-phase column (Aquapore RP-300 7 μ C8 220 x 4.6 mm, Applied Biosystems) 20 protected by a guard cartridge (RP-8, 15 x 3.2 mm, Applied Biosystems) and equilibrated to 40°C at 0.5 mL.minute. Protein was eluted under these conditions using the following programmed linear gradient:

time (min)	%B	Solvent A: 0.1% trifluoroacetic acid (TFA) Solvent B: 90% acetonitrile, 0.1% TFA
25 0		
60	66.6	
62.0	100	
72.0	100	
75.0	0	

30 200 μ L (0.4 minute) fractions were collected in siliconized tubes (Multilube tubes, Bioquote) from 15.2 minutes after the beginning of the programmed gradient.

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VI. SDS-Polyacrylamide Gel Electrophoresis

In this study, protein molecular weight standards, low range, catalogue no. 161-0304, from Bio-Rad Laboratories Limited, Watford, England were employed. The actual 5 proteins used, and their molecular weight standards, have been listed herein previously.

Fractions 47 to 53 (GGF-I) and fractions 61 to 67 (GGFII) inclusive from the reversed-phase runs were individually pooled. 7 µL of the pooled material was boiled 10 in an equal volume of 0.0125 M Tris-Cl, 4% SDS, 20% glycerol, and 10% β -mercaptoethanol for GGF-I, for 5 minutes and loaded on to an 11% polyacrylamide Laemmli gel with a 4% stacking gel and run at a constant voltage of 50 V for 16 hours. This gel was then fixed and stained using a silver 15 staining kit (Amersham). Under these conditions, the factors are each seen as a somewhat diffuse band at relative molecular weights 30,000 to 36,000 Daltons (GGF-I) and 55,000 to 63,000 Daltons (GGFII) as defined by molecular weight markers. From the gel staining, it is apparent that 20 there are a small number of other protein species present at equivalent levels to the GGF-I and GGF-II species in the material pooled from the reversed-phase runs.

VII. Stability in Trifluoroacetic Acid

25 Stability data were obtained for the present Factors in the presence of trifluoroacetic acid, as follows:-

GGF-I: Material from the reversed-phase HPLC, in the presence of 0.1% TFA and acetonitrile, was assayed within 12 hours of the completion of the column run and then after 10 weeks incubation at 40°C. Following incubation, 30 the GGF-I had at least 50% of the activity of that material assayed directly off the column.

GGF-II: Material from the reversed-phase HPLC, in the presence of 0.1% TFA and acetonitrile, and stored at

-20°C, was assayed after thawing and then after 4 days incubation at 40°C. Following incubation, the GGF-II had at least 50% of the activity of that material freshly thawed.

It will be appreciated that the trifluoroacetic acid concentration used in the above studies is that most commonly used for reversed-phase chromatography.

VIII. Activity Assay Conditions

Unless otherwise indicated, all operations were conducted at 37°C, and, with reference to Figures 1 to 6, activity at each stage was determined using the Brookes (Meth. Enz., supra) techniques with the following modifications. Thus, in preparing Schwann cells, 5 µM forskolin was added in addition to DMEM (Dulbecco's modified Eagle's medium), FCS and GGF. Cells used in the assay were fibroblast-free Schwann cells at passage number less than 10, and these cells were removed from flasks with trypsin and plated into flat-bottomed 96-well plates at 3.3 thousand cells per microwell.

[¹²⁵I]IUDR was added for the final 24 hours after the test solution addition. The background (unstimulated) incorporation to each assay was less than 100 cpm, and maximal incorporation was 20 to 200 fold over background depending on Schwann cell batch and passage number.

In the case of the GGF-I and GGF-II fractions from reversed-phase HPLC as described above, two dose response curves were also produced for each factor, using exactly the above method for one of the curves for each factor, and the above method modified in the assay procedure only by substituting foetal calf plasma for fetal calf serum to obtain the other curve for each factor. The results are in Figures 7 and 8.

EXAMPLE 2

Amino acid sequences of purified GGF-I and GGF-II

Amino acid sequence analysis studies were performed using highly purified bovine pituitary GGF-I and GGF-II.

- 5 The conventional single letter code was used to describe the sequences. Peptides were obtained by lysyl endopeptidase and protease V8 digests, carried out on reduced and carboxymethylated samples, with the lysyl endopeptidase digest of GGF-II carried out on material eluted from the
10 55-65 RD region of a 11% SDS-PAGE (MW relative to the above-quoted markers).

A total of 21 peptide sequences (see Figure 9, SEQ ID Nos. 1-20, 169) were obtained for GGF-I, of which 12 peptides (see Figure 10, SEQ ID Nos. 1, 22-29, 17, 19, and
15 32) are not present in current protein databases and therefore represent unique sequences. A total of 12 peptide sequences (see Figure 11, SEQ ID Nos. 33-44) were obtained for GGF-II, of which 10 peptides (see Figure 12, SEQ ID Nos.
20 45-53) are not present in current protein databases and therefore represent unique sequences (an exception is peptide GGF-II 06 which shows identical sequences in many proteins which are probably of no significance given the small number of residues). These novel sequences are extremely likely to correspond to portions of the true amino
25 acid sequences of GGFs I and II.

Particular attention can be drawn to the sequences of GGF-I 07 and GGF-II 12, which are clearly highly related. The similarities indicate that the sequences of these peptides are almost certainly those of the assigned GGF species, and are most unlikely to be derived from contaminant proteins.

In addition, in peptide GGF-II 02, the sequence X S
S is consistent with the presence of an N link d
carbohydrate moiety in an asparagine at the position denoted
by X.

5 In general, in Figures 9 and 11, X represents an
unknown residue denoting a sequencing cycle where a single
position could not be called with certainty either because
there was more than one signal of equal size in the cycle or
because no signal was present. As asterisk denotes those
10 peptides where the last amino acid called corresponds to the
last amino acid present in that peptide. In the remaining
peptides, the signal strength after the last amino acid
called was insufficient to continue sequence calling to the
end of that peptide. The right hand column indicates the
15 results of a computer database search using the GCG package
FASTA and TFASTA programs to analyze the NBRF and EMBL
sequence databases. The name of a protein in this column
denotes identity of a portion of its sequence with the
peptide amino acid sequence called allowing a maximum of two
20 mismatches. A question mark denotes three mismatches
allowed. The abbreviations used are as follows:

HMG-1	High Mobility Group protein-1
HMG-2	High Mobility Group protein-2
LH-alpha	Luteinizing hormone alpha subunit
25 LH-beta	Luteinizing hormone beta subunit

EXAMPLE 3

Mitogenic Activity of Purified GGF-I and GGF-II

The mitogenic activity of a highly purified sample containing both GGFs I and II was studied using a quantitative method, which allows a single microculture to be examined for DNA synthesis, cell morphology, cell number and expression of cell antigens. This technique has been modified from a method previously reported by Muir et al., Analytical Biochemistry 185, 377-382, 1990. The main modifications are: 1) the use of uncoated microtiter plates, 2) the cell number per well, 3) the use of 5% Foetal Bovine Plasma (FBP) instead of 10% Foetal Calf Serum (FCS), and 4) the time of incubation in presence of mitogens and bromodeoxyuridine (BrdU), added simultaneously to the cultures. In addition the cell monolayer was not washed before fixation to avoid loss of cells, and the incubation time of monoclonal mouse anti-BrdU antibody and peroxidase conjugated goat anti-mouse immunoglobulin (IgG) antibody were doubled to increase the sensitivity of the assay. The assay, optimized for rat sciatic nerve Schwann cells, has also been used for several cell lines, after appropriate modifications to the cell culture conditions.

I. Methods of Mitogenesis Testing

On day 1, purified Schwann cells were plated onto uncoated 96 well plates in 5% FBP/Dulbecco's Modified Eagle Medium (DMEM) (5,000 cells/well). On day 2, GGFs or other test factors were added to the cultures, as well as BrdU at a final concentration of 10 μ M. After 48 hours (day 4) BrdU incorporation was terminated by aspirating the medium and cells were fixed with 200 μ l/well of 70% ethanol for 20 min at room temperature. Next, the cells were washed with water

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and the DNA denatured by incubation with 100 μ l 2N HCl for 10 min at 37°C. Following aspiration, residual acid was neutralized by filling the wells with 0.1 M borate buffer, pH 9.0, and the cells were washed with phosphate buffer and saline (PBS). Cells were then treated with 50 μ l of blocking buffer (PBS containing 0.1% Triton X-100 and 2% normal goat serum) for 15 min at 37°C. After aspiration, monoclonal mouse anti-BrdU antibody (Dako Corp., Santa Barbara, CA) (50 μ l/well, 1.4 μ g/ml diluted in blocking buffer) was added and incubated for two hours at 37°C. Unbound antibodies were removed by three washes in PBS containing 0.1% Triton X-100 and peroxidase-conjugated goat anti-mouse IgG antibody (Dako Corp., Santa Barbara, CA) (50 μ l/well, 2 μ g/ml diluted in blocking buffer) was added and incubated for one hour at 37°C. After three washes in PBS/Triton and a final rinse in PBS, wells received 100 μ l/well of 50 mM phosphate/citrate buffer, pH 5.0, containing 0.05% of the soluble chromogen o-phenylenediamine (OPD) and 0.02% H_2O_2 . The reaction was terminated after 5-20 min at room temperature, by pipetting 80 μ l from each well to a clean plate containing 40 μ l/well of 2N sulfuric acid. The absorbance was recorded at 490nm using a plate reader (Dynatech Labs). The assay plates containing the cell monolayers were washed twice with PBS and immunocytochemically stained for BrdU-DNA by adding 100 μ l/well of the substrate diaminobenzidine (DAB) and 0.02% H_2O_2 to generate an insoluble product. After 10-20 min the staining reaction was stopped by washing with water, and BrdU-positive nuclei observed and counted using an inverted microscope. Occasionally, negative nuclei were counterstained with 0.001% Toluidine blue and counted as before.

II. Cell lines used for Mitogenesis Assays

Swiss 3T3 Fibroblasts: Cells, from Flow Labs, were maintained in DMEM supplemented with 10% FCS, penicillin and streptomycin, at 37°C in a humidified atmosphere of 10% CO₂ in air. Cells were fed or subcultured every two days. For mitogenic assay, cells were plated at a density of 5,000 cells/well in complete medium and incubated for a week until cells were confluent and quiescent. The serum containing medium was removed and the cell monolayer washed twice with serum free-medium. 100 µl of serum free medium containing mitogens and 10µM of BrdU were added to each well and incubated for 48 hours. Dose responses to GGFs and serum or PDGF (as a positive control) were performed.

15 BEK (Baby Hamster Kidney) 21 C13 Fibroblasts: Cells from European Collection of Animal Cell Cultures (ECACC), were maintained in Glasgow Modified Eagle Medium (GMEM) supplemented with 5% tryptose phosphate broth, 5% FCS, penicillin and streptomycin, at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells were fed or subcultured 20 every two to three days. For mitogenic assay, cells were plated at a density of 2,000 cell/well in complete medium for 24 hours. The serum containing medium was then removed and after washing with serum free medium, replaced with 100 µl of 0.1% FCS containing GMEM or GMEM alone. GGFs and FCS 25 or bFGF as positive controls were added, coincident with 10µM BrdU, and incubated for 48 hours. Cell cultures were then processed as described for Schwann cells.

C6 Rat Glioma Cell Line: Cells, obtained at passage 39, were maintained in DMEM containing 5% FCS, 5% Horse 30 serum (HS), penicillin and streptomycin, at 37°C in a humidified atmosphere of 10% CO₂ in air. Cells were fed or

subcultured every three days. For mitogenic assay, cells were plated at a density of 2,000 cells/well in complete medium and incubated for 24 hours. Then medium was replaced with a mixture of 1:1 DMEM and F12 medium containing 0.1% FCS, after washing in serum free medium. Dose responses to GGFs, FCS and cFGF were then performed and cells were processed through the ELISA as previously described for the other cell types.

PC12 (Rat Adrenal Pheochromocytoma Cells): Cells from ECACC, were maintained in RPMI 1640 supplemented with 10% HS, 5% FCS, penicillin and streptomycin, in collagen coated flasks, at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells were fed every three days by replacing 80% of the medium. For mitogenic assay, cells were plated at a density of 3,000 cells/well in complete medium, on collagen coated plates (50 µl/well collagen, Vitrogen Collagen Corp., diluted 1 : 50, 30 min at 37°C) and incubated for 24 hours. The medium was then placed with fresh RPMI either alone or containing 1 mM insulin or 1% FCS. Dose responses to FCS/HS (1:2) as positive control and to GGFs were performed as before. After 48 hours cells were fixed and the ELISA performed as previously described.

III. Results of Mitogenesis Assays: All the experiments presented in this Example were performed using a highly purified sample from a Sepharose 12 chromatography purification step (see Example 1, section D) containing a mixture of GGF-I and GGF-II (GGFs).

First, the results obtained with the BrdU incorporation assay were compared with the classical mitogenic assay for Schwann cells based on [¹²⁵I]-UdR

incorporation into DNA of dividing cells, described by J.P.Brock et al (Methods Enzymol. 147:217, 1987).

Figure 13 shows the comparison of data obtained with the two assays, performed in the same cell culture conditions (5,000 cells/well, in 5% FBS/DMEM, incubated in presence of GGFs for 48hrs). As clearly shown, the results are comparable, but BrdU incorporation assay appears to be slightly more sensitive, as suggested by the shift of the curve to the left of the graph, i.e. to lower concentrations of GGFs.

As described under the section "Methods of Mitogenesis Testing", after the immunoreactive BrdU-DNA has been quantitated by reading the intensity of the soluble product of the OPD peroxidase reaction, the original assay plates containing cell monolayers can undergo the second reaction resulting in the insoluble DAB product, which stains the BrdU positive nuclei. The microcultures can then be examined under an inverted microscope, and cell morphology and the numbers of BrdU-positive and negative nuclei can be observed.

In Figure 14a and Figure 14b the BrdU-DNA immunoreactivity, evaluated by reading absorbance at 490 nm, is compared to the number of BrdU-positive nuclei and to the percentage of BrdU-positive nuclei on the total number of cells per well, counted in the same cultures. Standard deviations were less than 10%. The two evaluation methods show a very good correlation and the discrepancy between the values at the highest dose of GGFs can be explained by the different extent of DNA synthesis in cells detected as BrdU-positive.

The BrdU incorporation assay can therefore provide additional useful information about the biological activity of polypeptides on Schwann cells when compared to the (125)

I-UdR incorporation assay. For example, the data reported in Figure 15 show that GGFs can act on Schwann cells to induce DNA synthesis, but at low doses to increase the number of negative cells present in the microculture after 48 hours.

The assay has then been used on several cell lines of different origin. In Figure 16 the mitogenic responses of Schwann cells and Swiss 3T3 fibroblasts to GGFs are compared; despite the weak response obtained in 3T3 fibroblasts, some clearly BrdU-positive nuclei were detected in these cultures. Control cultures were run in parallel in presence of several doses of FCS or human recombinant PDGF, showing that the cells could respond to appropriate stimuli (not shown).

The ability of fibroblasts to respond to GGFs was further investigated using the BHK 21 C13 cell line. These fibroblasts, derived from kidney, do not exhibit contact inhibition or reach a quiescent state when confluent. Therefore the experimental conditions were designed to have a very low background proliferation without compromising the cell viability. GGFs have a significant mitogenic activity on BHK21 C13 cells as shown by Figure 17 and Figure 18. Figure 17 shows the BrdU incorporation into DNA by BHK 21 C13 cells stimulated by GGFs in the presence of 0.1% FCS. The good mitogenic response to FCS indicates that cell culture conditions were not limiting. In Figure 18 the mitogenic effect of GGFs is expressed as the number of BrdU-positive and BrdU-negative cells and as the total number of cells counted per well. Data are representative of two experiments run in duplicates; at least three fields per well were counted. As observed for Schwann cells in addition to a proliferative effect at low doses, GGFs also increase the numbers of nonresponding cells surviving. The

percentage of BrdU positive cells is proportional to the increasing amounts of GGFs added to the cultures. The total number of cells after 48 hours in presence of higher doses of GGFs is at least doubled, confirming that GGFs induce DNA synthesis and proliferation in BHK21 C13 cells. Under the same conditions, cells maintained for 48 hours in the presence of 2% FCS showed an increase of about six fold (not shown).

C6 glioma cells have provided a useful model to study glial cell properties. The phenotype expressed seems to be dependent on the cell passage, the cells more closely resembling an astrocyte phenotype at an early stage, and an oligodendrocyte phenotype at later stages (beyond passage 70). C6 cells used in these experiments were from passage 39 to passage 52. C6 cells are a highly proliferating population, therefore the experimental conditions were optimized to have a very low background of BrdU incorporation. The presence of 0.1% serum was necessary to maintain cell viability without significantly affecting the mitogenic responses, as shown by the dose response to FCS (Figure 19).

In Figure 20 the mitogenic responses to aFGF (acidic Fibroblast growth factor) and GGFs are expressed as the percentages of maximal BrdU incorporation obtained in the presence of FCS (8%). Values are averages of two experiments, run in duplicates. The effect of GGFs was comparable to that of a pure preparation of aFGF. aFGF has been described as a specific growth factor for C6 cells (Lim R. et al., Cell Regulation 1:741-746, 1990) and for that reason it was used as a positive control. The direct counting of BrdU positive and negative cells was not possible because of the high cell density in the microcultures. In contrast to the cell lines so far

reported, PC12 cells did n t show any vident resp nsiveness to GGFS, wh n tr ated und r cultur conditions in which PC12 could r spond to sera (mixture of FCS and HS as used routinely for cell maintenance). Nevertheless the number of 5 cells plated per well seems to affect the behavior of PC12 cells, and therefore further experiments are required.

EXAMPLE 4

Isolating and Cloning of Nucleotide Sequences encoding proteins containing GGF-I and GGF-II peptides

10 Isolation and cloning of the GGF-II nucleotide sequences was performed as outlined herein, using peptide sequence information and library screening, and was performed as set out below. It will be appreciated that the peptides of Figures 4 and 5 can be used as the starting 15 point for isolation and cloning of GGF-I sequences by following the techniques described herein. Indeed, Figure 21, SEQ ID Nos. 54-88) shows possible degenerate oligonucleotide probes for this purpose, and Figure 23, SEQ ID Nos. 90-119, lists possible PCR primers. DNA sequence 20 and polypeptide sequence should be obtainable by this means as with GGF-II, and also DNA constructs and expression vectors incorporating such DNA sequence, host cells genetically altered by incorporating such constructs/vectors, and protein obtainable by cultivating 25 such host cells. The invention envisages such subject matter.

I. Design and Synthesis of oligonucleotide Probes and Primers

Degenerate DNA oligomer probes were designed by 30 backtranslating the amino acid sequences (derived from the peptides generated from purified GGF protein) into

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nucleotide sequences. Oligomers represented either the coding strand or the non-coding strand of the DNA sequence. When serine, arginine or leucine were included in the oligomer design, then two separate syntheses were prepared to avoid ambiguities. For example, serine was encoded by either TCN or AGY as in 537 and 538 or 609 and 610. Similar codon splitting was done for arginine or leucine (e.g. 544, 545). DNA oligomers were synthesized on a Biosearch 8750 4-column DNA synthesizer using β -cyanoethyl chemistry operated at 0.2 micromole scale synthesis. Oligomers were cleaved off the column (500 angstrom CpG resins) and deprotected in concentrated ammonium hydroxide for 6-24 hours at 55-60°C. Deprotected oligomers were dried under vacuum (Speedvac) and purified by electrophoresis in gels of 15% acrylamide (20 mono : 1 bis), 50 mM Tris-borate-EDTA buffer containing 7M urea. Full length oligomers were detected in the gels by UV shadowing, then the bands were excised and DNA oligomers eluted into 1.5 ml H₂O for 4-16 hours with shaking. The eluate was dried, redissolved in 0.1 ml H₂O and absorbance measurements were taken at 260nm.

Concentrations were determined according to the following formula:

$$(A_{260} \times \text{units/ml}) (60.6/\text{length} = x \mu\text{M})$$

All oligomers were adjusted to 50 μM concentration by addition of H₂O.

Degenerate probes designed as above are shown in Figure 21, SEQ ID Nos. 54-88.

PCR primers were prepared by essentially the same procedures that were used for probes with the following modifications. Linkers of thirteen nucleotides containing restriction sites were included at the 5' ends of the degenerate oligomers for use in cloning into vectors. DNA synthesis was performed at 1 micromole scale using 1,000

angstrom CpG resins and inosine was used at positions where all four nucleotides were incorporated normally into degenerate probes. Purifications of PCR primers included an ethanol precipitation following the gel electrophoresis 5 purification.

II. Library Construction and Screening

A bovine genomic DNA library was purchased from Stratagene (Catalogue Number: 945701). The library contained 2×10^6 15-20kb Sau3AI partial bovine DNA fragments cloned into the vector lambda DashII. A bovine total brain cDNA library was purchased from Clonetech (Catalogue Number: BL 10139). Complementary DNA libraries were constructed (In Vitrogen; Stratagene) from mRNA prepared from bovine total brain, from bovine pituitary and 10 from bovine posterior pituitary. In Vitrogen prepared two cDNA libraries: one library was in the vector lambda g10, the other in vector pCDNAI (a plasmid library). The Stratagene libraries were prepared in the vector lambda unizap. Collectively, the cDNA libraries contained 14 15 million primary recombinant phage.

The bovine genomic library was plated on *E. coli* K12 host strain LE392 on 23 x 23 cm plates (Nunc) at 150,000 to 200,000 phage plaques per plate. Each plate represented approximately one bovine genome equivalent. Following an 20 overnight incubation at 37°C, the plates were chilled and replicate filters were prepared according to procedures of Maniatis et al. (2:60-81). Four plaque lifts were prepared 25 from each plate onto uncharged nylon membranes (Pall Biodyne A or MSI Nitropure). The DNA was immobilized onto the membranes by cross-linking under UV light for 5 minutes or, 30 by baking at 80°C under vacuum for two hours. DNA probes were labelled using T4 polynucleotide kinase (New England

Biolabs) with gamma ^{32}P ATP (N w England Nuclear; 6500 Ci/mmol) according to the specifications of the suppli rs. Briefly, 50 pmols of d generate DNA oligomer were incubated in the presence of 600 μCi gamma ^{32}P -ATP and 5 units T4 5 polynucleotide kinase for 30 minutes at 37°C. Reactions were terminated, gel electrophoresis loading buffer was added and then radiolabelled probes were purified by electrophoresis. ^{32}P labelled probes were excised from gel slices and eluted into water. Alternatively, DNA probes 10 were labelled via PCR amplification by incorporation of α - ^{32}P -dATP or α - ^{32}P dCTP according to the protocol of Schowalter and Sommer, Anal. Biochem 177:90-94 (1989). Probes labelled in PCR reactions were purified by desalting 15 on Sephadex G-150 columns.

Prehybridization and hybridization were performed in GMC buffer (0.52 M NaPi, 7% SDS, 1% BSA, 1.5 mM EDTA, 0.1 M NaCl 10 mg/ml tRNA). Washing was performed in oligowash (160 ml 1 M Na_2HPO_4 , 200 ml 20% SDS, 8.0 ml 0.5 M EDTA, 100 ml 5M NaCl, 3632 ml H₂O). Typically, 20 filters (400 sq. 20 centimeters each) representing replicate copies of ten bovine genome equivalents were incubated in 200 ml hybridization solution with 100 pmols of degenerate oligonucleotide probe (128-512 fold degenerate).

Hybridization was allowed to occur overnight at 5°C below 25 the minimum melting temperature calculated for the degenerate probe. The calculation of minimum melting temperature assumes 2°C for an AT pair and 4°C for a GC pair.

Filters were washed in repeated changes of oligowash 30 at the hybridization temperatures four to five hours and finally, in 3.2M tetramethylammonium chloride, 1% SDS twice for 30 min at a temperature dependent on the DNA probe length. For 20mers, the final wash temperature was 60°C.

Filters were mounted, then exposed to X-ray film (Kodak XAR5) using intensifying screens (Dupont Cron x Lightening Plus). Usually, a three to five day film exposure at minus 80°C was sufficient to detect duplicate signals in the library screens. Following analysis of the results, filters could be stripped and reprobed. Filters were stripped by incubating through two successive cycles of fifteen minutes in a microwave oven at full power in a solution of 1% SDS containing 10mM EDTA pH8. Filters were taken through at least three to four cycles of stripping and reprobing with various probes.

III. Recombinant Phage Isolation, Growth and DNA Preparation

These procedures followed standard protocol as described in Recombinant DNA (Maniatis et al 2:60-2:81).

IV. Analysis of Isolated Clones Using DNA Digestion and Southern Blots

Recombinant Phage DNA samples (2 micrograms) were digested according to conditions recommended by the restriction endonuclease supplier (New England Biolabs). Following a four hour incubation at 37°C, the reaction products were precipitated in the presence of 0.1M sodium acetate and three volumes of ethanol. Precipitated DNA was collected by centrifugation, rinsed in 75% ethanol and dried. All resuspended samples were loaded onto agarose gels (typically 1% in TAE buffer; 0.04M Tris acetate, 0.002M EDTA). Gel runs were at 1 volt per centimeter from 4 to 20 hours. Markers included lambda Hind III DNA fragments and/or λ X174HaeIII DNA fragments (New England Biolabs). The gels were stained with 0.5 micrograms/ml of ethidium bromide and photographed. For southern blotting, DNA was first

depurinated in the gel by treatment with 0.125 N HCl,
denatured in 0.5 N NaOH and transferred in 20x SSC (3M
sodium chloride, 0.03 M sodium citrate) to uncharged nylon
membranes. Blotting was done for 6 hours up to 24 hours,
then the filters were neutralized in 0.5 Tris HCl pH 7.5,
0.15 M sodium chloride, then rinsed briefly in 50 mM
Tris-borate EDTA.

For cross-linking, the filters were wrapped first in
transparent plastic wrap, then the DNA side exposed for five
minutes to an ultraviolet light. Hybridization and washing
was performed as described for library screening (see
section 2 of this Example). For hybridization analysis to
determine whether similar genes exist in other species
slight modifications were made. The DNA filter was
purchased from Clonetech (Catalogue Number 7753-1) and
contains 5 micrograms of EcoRI digested DNA from various
species per lane. The probe was labelled by PCR
amplification reactions as described in section 2 above, and
hybridizations were done in 80% buffer B (2 g
polyvinylpyrrolidine, 2 g Ficoll-400, 2 g bovine serum
albumin, 50 ml 1M Tris-HCl (pH 7.5) 58 g NaCl, 1 g sodium
pyrophosphate, 10 g sodium dodecyl sulfate, 950 ml H₂O)
containing 10% dextran sulfate. The probes were denatured
by boiling for ten minutes then rapidly cooling in ice
water. The probe was added to the hybridization buffer at
 10^6 dpm ³²P per ml and incubated overnight at 60°C. The
filters were washed at 60°C first in buffer B followed by 2X
SSC, 0.1% SDS then in 1x SSC, 0.1% SDS. For high
stringency, experiments, final washes were done in 0.1 x
SSC, 1% SDS and the temperature raised to 65°C.

Southern blot data were used to prepare a
restriction map of the genomic clone and to indicate which

subfragments hybridized to the GGF probes (candidates for subcloning).

V. Subcloning of Segments of DNA Homologous to Hybridization Probes

- 5 DNA digests (e.g. 5 micrograms) were loaded onto 1% agarose gels then appropriate fragments excised from the gels following staining. The DNA was purified by adsorption onto glass beads followed by elution using the protocol described by the supplier (Bio 101). Recovered DNA
10 fragments (100-200 ng) were ligated into linearized dephosphorylated vectors, e.g. pT3T7 (Ambion), which is a derivative of pUC18, using T4 ligase (New England Biolabs). This vector carries the *E. coli* β lactamase gene, hence, transformants can be selected on plates containing
15 ampicillin. The vector also supplies β -galactosidase complementation to the host cell, therefore non-recombinants (blue) can be detected using isopropylthiogalactoside and Bluogal (Bethesda Research Labs). A portion of the ligation reactions was used to transform *E. coli* K12 XL1 blue
20 competent cells (Stratagene Catalogue Number: 200236) and then the transformants were selected on LB plates containing 50 micrograms per ml ampicillin. White colonies were selected and plasmid mini preps were prepared for DNA digestion and for DNA sequence analysis. Selected clones
25 were retested to determine if their insert DNA hybridized with the GGF probes.

VI. DNA Sequencing

Double stranded plasmid DNA templates were prepared from 5 ml cultures according to standard protocols.

- 30 Sequencing was by the dideoxy chain termination method using Sequenase 2.0 and a dideoxynucleotide sequencing kit (US

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- Biochemical) according to the manufacturers protocol (a modification of Sanger et al. PNAS; USA 74:5463 (1977)). Alternatively, sequencing was done in a DNA thermal cycle (Perkin Elmer, model 4800) using a cycle sequencing kit (New England Biolabs; Bethesda Research Laboratories) and was performed according to manufacturers instructions using a 5'-end labelled primer. Sequence primers were either those supplied with the sequencing kits or were synthesized according to sequence determined from the clones.
- 5 10 Sequencing reactions were loaded on and resolved on 0.4mm thick sequencing gels of 6% polyacrylamide. Gels were dried and exposed to X-Ray film. Typically, 35 S was incorporated when standard sequencing kits were used and a 32 P end labelled primer was used for cycle sequencing reactions.
- 15 Sequences were read into a DNA sequence editor from the bottom of the gel to the top (5' direction to 3') and data were analyzed using programs supplied by Genetics Computer Group (GCG, University of Wisconsin).

VII. RNA Preparation and PCR Amplification

- 20 Open reading frames detected in the genomic DNA and which contained sequence encoding GGF peptides were extended via PCR amplification of pituitary RNA. RNA was prepared from frozen bovine tissue (Pelfreeze) according to the guanidine neutral-CsCl procedure (Chirgwin et. al. Biochemistry 18:5294(1979).) Polyadenylated RNA was selected by oligo-dT cellulose column chromatography (Aviv and Leder PNAS (USA) 69:1408 (1972)).
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Specific DNA target sequences were amplified beginning with either total RNA or polyadenylated RNA samples that had been converted to cDNA using the Perkin Elmer PCR/RNA Kit Number: N808-0017. First strand reverse transcription reactions used 1 μ g template RNA and either

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primers of oligo dT with restriction enzyme recognition site linkers attach the specific antisense primers determined from cloned sequences with restriction sites attached. To produce the second strand, other primers plus 5 strand unique sequences as used in 3' RACE reactions (Froehman et al., PNAS (USA) 85:8998 (1988)) or were oligo dT primers with restriction sites attached if the second target site had been added by terminal transferase tailing first strand reaction products with dATP (e.g. 5' race 10 reactions, Froehman et al., ibid). Alternatively, as in anchored PCR reactions the second strand primers were degenerate, hence, representing particular peptide sequences.

The amplification profiles followed the following 15 general scheme: 1) five minutes soak file at 95°C; 2) thermal cycle file of 1 minute, 95°C; 1 minute ramped down to an annealing temperature of 45°C, 50°C or 55°C; maintain the annealing temperature for one minute; ramp up to 72°C over one minute; extend at 72°C for one minute or for one 20 minute plus a 10 second auto extension; 3) extension cycle at 72°C, five minutes, and; 4) soak file 4°C for infinite time. Thermal cycle files (#2) usually were run for 30 cycles. A sixteen μl sample of each 100 μl amplification 25 reaction was analyzed by electrophoresis in 2% NuSieve 1% agarose gels run in TAE buffer at 4 volts per centimeter for three hours. The gels were stained, then blotted to uncharged nylon membranes which were probed with labelled DNA probes that were internal to the primers.

Specific sets of DNA amplification products could be 30 identified in the blotting experiments and their positions used as a guide to purification and reamplification. When appropriate, the remaining portions of selected samples were loaded onto preparative gels, then following electrophoresis

four to five slices of 0.5 mm thickness (bracketing the expected position of the specific product) were taken from the gel. The agarose was crushed, then soaked in 0.5 ml of electrophoresis buffer from 2-16 hours at 40°C. The crushed agarose was centrifuged for two minutes and the aqueous phase was transferred to fresh tubes.

Reamplification was done on five microliters (roughly 1% of the product) of the eluted material using the same sets of primers and the reaction profiles as in the original reactions. When the reamplification reactions were completed, samples were extracted with chloroform and transferred to fresh tubes. Concentrated restriction enzyme buffers and enzymes were added to the reactions in order to cleave at the restriction sites present in the linkers. The digested PCR products were purified by gel electrophoresis, then subcloned into vectors as described in the subcloning section above. DNA sequencing was done described as above.

VIII. DNA Sequence Analysis

DNA sequences were assembled using a fragment assembly program and the amino acid sequences deduced by the GCG programs GelAssemble, Map and Translate. The deduced protein sequences were used as a query sequence to search protein sequence databases using WordSearch. Analysis was done on a VAX Station 3100 workstation operating under VMS 5.1. The database search was done on SwissProt release number 21 using GCG Version 7.0.

IX. Results of Cloning and Sequencing of genes encoding GGF-I and GGF-II

As indicated above, to identify the DNA sequence encoding bovine GGF-II degenerate oligonucleotide probes

WERE DESIGNATED FROM GGF-II PEPTIDE SEQUENCES. GGF-II 12 (SEQ ID NO. 44), A PEPTIDE GENERATED VIA LYSYL ENDOPEPTIDASE DIGESTION OF A PURIFIED GGF-II PREPARATION (SEE FIGURES 11 AND 12) SHOWED STRONG AMINO ACID SEQUENCE HOMOLOGY WITH GGF-I 07 (SEQ ID NO. 39), A TRYPTIC PEPTIDE GENERATED FROM A PURIFIED GGF-I PREPARATION. GGF-II 12 WAS THUS USED TO CREATE TEN DEGENERATE OLIGONUCLEOTIDE PROBES (SEE OLIGOS 609, 610 AND 649 TO 656 IN FIGURE 21, SEQ ID NOS. 69, 70, 71 AND 79, RESPECTIVELY). A DUPLICATE SET OF FILTERS WERE PROBED WITH TWO SETS (SET 1=609, 610; SET 2=649-656) OF PROBES ENCODING TWO OVERLAPPING PORTIONS OF GGF-II 12. HYBRIDIZATION SIGNALS WERE OBSERVED, BUT, ONLY ONE CLONE HYBRIDIZED TO BOTH PROBE SETS. THE CLONE (DESIGNATED GGF2BG1) WAS PURIFIED.

SOUTHERN BLOTH ANALYSIS OF DNA FROM THE PHAGE CLONE GGF2BG1 CONFIRMED THAT BOTH SETS OF PROBES HYBRIDIZED WITH THAT BOVINE DNA SEQUENCE, AND SHOWED FURTHER THAT BOTH PROBES REACTED WITH THE SAME SET OF DNA FRAGMENTS WITHIN THE CLONE. BASED ON THOSE EXPERIMENTS A 4 KB ECO RI SUB-FRAGMENT OF THE ORIGINAL CLONE WAS IDENTIFIED, SUBCLONED AND PARTIALLY SEQUENCED. FIGURE 22 SHOWS THE NUCLEOTIDE SEQUENCE, SEQ ID NO. 89) AND THE DEDUCED AMINO ACID SEQUENCE OF THE INITIAL DNA SEQUENCE READINGS THAT INCLUDED THE HYBRIDIZATION SITES OF PROBES 609 AND 650, AND CONFIRMED THAT A PORTION OF THIS BOVINE GENOMIC DNA ENCODED PEPTIDE 12 (SEQ ID NO. 188) (KASLADSGEYEM).

FURTHER SEQUENCE ANALYSIS DEMONSTRATED THAT GGF-II 12 RESIDED ON A 66 AMINO ACID OPEN READING FRAME (SEE BELOW) WHICH HAS BECOME THE STARTING POINT FOR THE ISOLATION OF OVERLAPPING SEQUENCES REPRESENTING A PUTATIVE BOVINE GGF-II GENE AND A cDNA.

Several PCR proc dur s wer used to obtain additional coding s quences for the putativ bovin GGF-II gene. Total RNA and ligo dT-sel ct d (poly A containing) RNA samples were prepar d from bovine total pituitary, 5 anterior pituitary, posterior pituitary, and hypothalamus. Using primers from the list shown in Figure 23, SEQ ID Nos. 109-119, one-sided PCR reactions (RACE) were used to amplify cDNA ends in both the 3' and 5' directions, and anchored PCR reactions were performed with degenerate oligonucleotide 10 primers representing additional GGF-II peptides. Figure 24 summarizes the contiguous DNA structures and sequences obtained in those experiments. From the 3' RACE reactions, three alternatively spliced cDNA sequences were produced, which have been cloned and sequenced. A 5' RACE reaction 15 led to the discovery of an additional exon containing coding sequence for at least 52 amino acids. Analysis of that deduced amino acid sequence revealed peptides GGF-II-6 and a sequence similar to GGF-I-18 (see below). The anchored PCR reactions led to the identification of (cDNA) coding 20 sequences of peptides GGF-II-1, 2, 3 and 10 contained within an additional cDNA segment of 300 bp. The 5' limit of this segment (i.e., segment E, see Fig. 31) is defined by the oligonucleotide which encodes peptide GGF-II-1 and which was used in the PCR reaction (additional 5' sequence data exists 25 as described for the human clone in Example 6). Thus this clone contains nucleotide sequences encoding six out of the existing total of nine novel GGF-II peptide sequences.

The cloned gene was characterized first by constructing a physical map of GGF2BG1 that allowed us to 30 position the coding sequences as they were found (see below, Figure 25). DNA probes from the coding sequences described above have been used to identify further DNA fragments containing the exons on this phage clone and to

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identify clones that overlap in both directions. The putative bovine GGF-II gene is divided into at least 5 coding segments. Coding segments are defined as discrete lengths of DNA sequence which can be translated into polypeptide sequences using the universal genetic code. The coding segments described in Figure 31 and referred to in the present application are: 1) particular exons present within the GGF gene (e.g. coding segment A), or 2) derived from sets of two or more exons that appear in specific sub-groups of mRNAs, where each set can be translated into the specific polypeptide segments as in the gene products shown. The polypeptide segments referred to in the claims are the translation products of the analogous DNA coding segments. Only coding segments A and B have been defined as exons and sequenced and mapped thus far. The summary of the contiguous coding sequences identified is shown in Figure 26. The exons are listed (alphabetically) in the order of their discovery. It is apparent from the intron/exon boundaries that exon B may be included in cDNAs that connect coding segment E and coding segment A. That is, exon B cannot be spliced out without compromising the reading frame. Therefore, we suggest that three alternative splicing patterns can produce putative bovine GGF-II cDNA sequences 1, 2 and 3. The coding sequences of these, designated GGF2BPP1.CDS, GGF2BPP2.CDS and GGF2BPP3.CDS, respectively, are given in Figures 28a (SEQ ID No. 133), 28b (SEQ ID No. 134), and 28c (SEQ ID No. 135), respectively. The deduced amino acid sequence of the three cDNAs is also given in Figures 28a, (SEQ ID No. 133), 28b (SEQ ID No. 134), and 28c (SEQ ID No. 135).

The three deduced structures encode proteins of lengths 206, 281 and 257 amino acids. The first 183 residues of the deduced protein sequence are identical in

all three gene products. At position 184 the clones differ significantly. A codon for glycine GGT in GGF2BPP1 also serves as a splice donor for GGF2BPP2 and GGF2BPP3, which alternatively add on exons C, C/D, C/D' and D or C, C/D and D, respectively, and shown in figure 33, SEQ ID No. 149).
5 GGFIIIBPP1 is a truncated gene product which is generated by reading past the coding segment A splice junction into the following intervening sequence (intron). This represents coding segment A' in figure 31 (SEQ ID No. 140). The transcript ends adjacent to a canonical AATAAA
10 polyadenylation sequence, and we suggest that this truncated gene product represents a bona fide mature transcript. The other two longer gene products share the same 3' untranslated sequence and polyadenylation site.

15 All three of these molecules contain six of the nine novel GGF-II peptide sequences (see Figure 12) and another peptide is highly homologous to GGF-I-18 (see Figure 27). This finding gives a high probability that this recombinant molecule encodes at least a portion of bovine GGF-II.
20 Furthermore, the calculated isoelectric points for the three peptides are consistent with the physical properties of GGF-I and II. Since the molecular size of GGF-II is roughly 60 kD, the longest of the three cDNAs should encode a protein with nearly one-half of the predicted number of
25 amino acids.

A probe encompassing the B and A exons was labelled via PCR amplification and used to screen a cDNA library made from RNA isolated from bovine posterior pituitary. One clone (GGF2BPP5) showed the pattern indicated in figure 30 and contained an additional DNA coding segment (G) between coding segments A and C. The entire nucleic acid sequence is shown in figure 32 (SEQ ID No. 148). The predicted translation product from the longest open reading frame is

241 amino acids. A portion of a second cDNA (GGF2BPP4) was also isolated from the bovine posterior pituitary library using the probe described above. This clone showed the pattern indicated in figure 30. This clone is incomplete at the 5' end, but is a splicing variant in the sense that it lacks coding segments G and D. BPP4 also displays a novel 3' end with regions H, K and L beyond region C/D. The sequence of BPP4 is shown in figure 34 (SEQ ID No. 150).

EXAMPLE 5

10 GGF Sequences in Various Species

Database searching has not revealed any meaningful similarities between any predicted GGF translation products and known protein sequences. This suggests that GGF-II is the first member of a new family or superfamily of proteins.

15 In high stringency cross hybridization studies (DNA blotting experiments) with other mammalian DNAs we have shown, clearly, that DNA probes from this bovine recombinant molecule can readily detect specific sequences in a variety of samples tested. A highly homologous sequence is also

20 detected in human genomic DNA. The autoradiogram is shown in figure 29. The signals in the lanes containing rat and human DNA represent the rat and human equivalents of the GGF gene, the sequences of several cDNA's encoded by this gene have been recently reported by Holmes et al. (Science 256: 25 1205 (1992)) and Wen et al. (Cell 69: 559 (1992)).

EXAMPLE 6

Isolation of a Human Sequence Encoding Human GGF2

Several human clones containing sequences from the bovine GGFII coding segment E were isolated by screening a human 30 cDNA library prepared from brain stem (Stratagene catalog #935206). This strategy was pursued based on the strong

link between most of the GGF2 peptides (unique to GF2) and those predicted peptide sequence from library containing the bovine E segment. This library was screened as described in Example 4, Section II using the oligonucleotide probes 914-

5 919 listed below.

(SEQ ID NO: 179)

C 914TCGGGCTCCATGAAGAAGATGTA_n

(SEQ ID NO: 180)

C 915TCCATGAAGAAGATGTACCTGCT

(SEQ ID NO: 181)

C 916ATGTACCTGCTGCTCCTCCTGTA_n

(SEQ ID NO: 181)

C 917TTGAAGAAGGACTCGCTGCTCA_n

(SEQ ID NO: 182)

C 918AAAGCCGGGGCTTGAAGAA_n

(SEQ ID NO: 181)

C 919ATGARGTGTGGCGCGAAA_n

(SEQ ID NO: 182)

Clones detected with these probes were further analyzed by hybridization. A probe derived from coding segment A (see Figure 21), which was produced by labeling a polymerase chain reaction (PCR) product from segment A, was also used to screen the primary library. Several clones that hybridized with both A and E derived probes were selected and one particular clone, GGF2HBS5, was selected for further analysis. This clone is represented by the pattern of coding segments (EBACC/D'D as shown in Figure 31). The E segment in this clone is the human equivalent of the truncated bovine version of E shown in Figure 37.

GGF2HBS5 is the most likely candidate to encode GGF-II of all the "putative" GGF-II candidates described. The length

of coding sequence segment E is 786 nucleotides plus 264 bases of untranslated sequence. The predicted size of the protein encoded by GGF2HBS5 is approximately 423 amino acids (approximately 45 kilodaltons, see Figure 45, SEQ ID NO:

C 170, which is similar to the size of the deglycosylated

form of GGF-II (see Example 16). Additionally, seven of the GGF-II peptides listed in Figure 27 have equivalent sequences which fall within the protein sequence predicted from region E. Pptides II-6 and II-12 are exceptions,

which fall in coding segment B and coding segment A, respectively. RNA encoding the GGF2HBS5 protein was produced in an *in vitro* transcription system driven by the bacteriophage T7 promoter resident in the vector (Bluescript SK [Stratagene Inc.] see Figure 44) containing the GGF2HBS5 insert. This RNA was translated in a cell free (rabbit reticulocyte) translation system and the size of the protein product was 45 Kd. Additionally, the cell-free product has been assayed in a Schwann cell mitogenic assay to confirm biological activity. Schwann cells treated with conditioned medium show both increased proliferation as measured by incorporation of 125 I-Uridine and phosphorylation on tyrosine of a protein in the 185 kilodalton range. Thus the size of the product encoded by GGF2HBS5 and the presence of DNA sequences which encode human peptides highly homologous to the bovine peptides shown in Figure 12 confirm that GGF2HBS5 encodes the human equivalent of bovine GGF2. The fact that conditioned media prepared from cells transformed with this clone elicits Schwann cell mitogenic activity confirms that the GGFIIHBS5 gene product (unlike the BPP5 gene product) is secreted. Additionally the GGFIIIBPP5 gene product seems to mediate the Schwann cell proliferation response via a receptor tyrosine kinase such as p185^{erbB2} or a closely related receptor (see Example 14).

25 EXAMPLE 7

Expression of Human Recombinant GGF2 in Mammalian and Insect Cells

The GGF2HBS5 cDNA clone encoding human GGF2 (as described in Example 6 and also referred to herein as HBS5) was cloned into vector pcDL-SRα296 (Takebe et al. Mol. Cell. Biol. 8:466-472 (1988) and COS-7 cells were transfected in 100 mm dishes by the DEAE-dextran method (Sambrook et al.

Molecular Cloning: A Laboratory Manual 2nd d. CSH
Laboratory NY (1989). Cell lysates r condition d m dia
from transiently xpr ssing COS ells w r harvested at 3 r
4 days post-transf cti n. To pr par lysat s, cell

5 monolayers were washed with PBS, scraped from the dishes,
lysed by three freeze/thaw cycles in 150 μ l of 0.25 M Tris-
HCl, pH 8. Cell debris was pelleted and the supernatant
recovered. Conditioned media samples (7 ml.) were
collected, then concentrated and buffer exchanged with 10 mM
10 Tris, pH 7.4 using Centiprep-10 and Centricon-10 units as
described by the manufacturer (Amicon, Beverly, MA). Rat
nerve Schwann cells were assayed for incorporation of DNA
synthesis precursors, as described (see Example 3).
Conditioned media or cell lysate samples were tested in the
15 Schwann cell proliferation assay as described in Example 3.
The mitogenic activity data are shown in Fig. 46. The cDNA,
GGF2HBS5, encoding GGF2 directed the secretion of the
protein product to the medium. A small proportion of total
activity was detectable inside the cells as determined by
20 assays using cell lysates. GGF2HFB1 and GGFBPP5 cDNA's
failed to direct the secretion of the product to the
extracellular medium. GGF activity from these clones was
detectable only in cell lysates (Fig. 46).

Recombinant GGF2 was also expressed in CHO cells.

25 The GGF2HBS5 cDNA encoding GGF2 was cloned into the EcoRI
site of vector pcdhfrpolyA (Fig. 54) and transfected into
the DHFR negative CHO cell line (DG44) by the calcium
phosphate coprecipitation method (Graham and Van Der Eb,
Virology 52:456-467 (1973). Clones were selected in
30 nucleotide and nucleoside free a medium (Gibco) in 96-well
plates. After 3 weeks, conditioned media samples from
individual clones were screened for expression of GGF by the
Schwann cell proliferation assay as described in Example 3.

Stable clones which secreted significant levels of GGF activity into the medium were identified. Schwann cell proliferation activity data from different volume aliquots of CHO cells conditioned medium were used to produce the dose response curve shown in Fig. 47 (ref., Graham and Van Der Eb, Virology 52:456, 1973). This material was analyzed on a Western blot probed with polyclonal antisera raised against a GGF2 specific peptide. A broad band of approximately 69-90 Kd (the expected size of GGF2 extracted from pituitary and higher molecular weight glycoforms) is specifically labeled (Fig. 49, lane 12).

Recombinant GGF2 was also expressed in insect cells using Baculovirus expression. Sf9 insect cells were infected with baculovirus containing the GGF2HBS5 cDNA clone at a multiplicity of 3-5 (10^6 cells/ml) and cultured in SF900-II medium (Gibco). Schwann cell mitogenic activity was secreted into the extracellular medium (Fig. 48). Different volumes of insect cell conditioned medium were tested in the Schwann cell proliferation assay in the absence of forskolin and the data used to produce the dose response curve shown in Fig. 48.

This material was also analyzed on a Western blot (Fig. 47) probed with the GGF II specific antibody described above. A band of 45 Kd, the size of deglycosylated GGF-II (see Example 16) was seen.

The methods used in this example were as follows: Schwann cell mitogenic activity of recombinant human and bovine glial growth factors was determined as follows: Mitogenic responses of cultured Schwann cells were measured in the presence of 5 μ M forskolin using crude recombinant GGF preparations obtained from transient mammalian expression experiments. Incorporation of [125 I]-Uridine was determined following an 18-24 hour exposure to materials

obtained from transfected or mock transfected COS cells as described in the Methods. The mean and standard deviation of four sets of data are shown. The mitogenic response to partially purified native bovine pituitary GGF
5 (carboxymethyl cellulose fraction; Goodearl et al., submitted) is shown (GGF) as a standard of one hundred percent activity.

cDNAs (Fig. 53) were cloned into pcDL-SRα296 (Takebe et al., Mol. Cell Biol. 8:466-472 (1988)), and COS-7 cells
10 were transfected in 100 mm dishes by the DEAE-dextran method (Sambrook et al., In Molecular Cloning. A Laboratory Manual, 2nd. ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989)). Cell lysates or conditioned media were harvested at 3 or 4 days post-transfection. To prepare
15 lysates, cell monolayers were washed with PBS, scraped from the dishes, and lysed by three freeze/thaw cycles in 150 µl of 0.25 M Tris-HCl, pH 8. Cell debris was pelleted and the supernate recovered. Conditioned media samples (7 mls) were collected, then concentrated and buffer exchanged with 10 mM
20 Tris, pH 7.4 using Centriprep-10 and Centricon-10 units as described by the manufacturer (Amicon, Beverly, MA). Rat sciatic nerve Schwann cells were assayed for incorporation of DNA synthesis precursors, as described (Davis and Stroobant, J. Cell Biol. 110:1353-1360 (1990); Brockes et
25 al., Brain Res. 165:105-118 (1979)).

Western blots of recombinant CHO cell conditioned medium were performed as follows: A recombinant CHO clone was cultured in 7 ml. of MCDB302 protein-free medium for 3 days. 2 ml of conditioned medium was concentrated, buffer exchanged against 10 mM Tris-HCl, pH 7.4 and lyophilized to dryness. The pellet was resuspended in SDS-PAGE sample buffer, subjected to reducing SDS gel electrophoresis and analyzed by Western blotting with a GGF peptide antibody. A

CHO control was done by using conditioned medium from untransfected CHO-DG44 host and the CHO HBS51 cells were assayed using conditioned medium from a recombinant clone.

EXAMPLE 8

5 Isolation of Other Human Sequences Related to Bovine GGF

The result in Examples 5 and 6 indicate that GGF related sequences from human sources can also be easily isolated by using DNA probes derived from bovine GGF sequences.

Alternatively the procedure described by Holmes et al.

10 (Science 256: 1205 (1992)) can be used. In this example a human protein (heregulin α), which binds to and activates the p185^{erbB2} receptor (and is related to GGF), is purified from a tumor cell line and the derived peptide sequence is used to produce oligonucleotide probes which were utilized 15 to clone the cDNA's encoding heregulin. The biochemical assay for p185^{erbB2} receptor activation is distinguished from Schwann cell proliferation. This is a similar approach to that used in examples 1-4 for the cloning of GGF sequences from pituitary cDNAs. The heregulin protein and 20 complementary DNAs were isolated from tumor cell lines according to the following procedures.

Heregulin was purified from medium conditioned by MDA-MB-231 breast cancer cells (ATCC #HTB 26) grown on Percell Biolytica microcarrier beads (Hyclone Labs). The medium (10 liters) was concentrated ~25-fold by filtration through a membrane (10-kD cutoff) (Millipore) and clarified by centrifugation and filtration through a filter (0.22 μm). The filtrate was applied to a heparin Sepharose column (Pharmacia) and the proteins were eluted with steps of 0.3, 30 0.6, and 0.9 M NaCl in phosphate-buffered saline. Activity in the various chromatographic fractions was measured by quantifying the increase in tyrosine phosphorylation of

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p185^{erbB2} in MCF-7 br ast tumor 115 (ATCC # HTB 22). MCF-7 cells were plated in 24-well Costar plates in F12 (50%) Dulbecco's minimum essential medium (50%) containing serum (10%) (10^5 cells per well), and allowed to attach for 5 at least 24 hours. Prior to assay, cells were transferred into medium without serum for a minimum of 1 hour. Column fractions (10 to 100 μ l) were incubated for 30 min. at 37°. Supernatants were then aspirated and the reaction was stopped by the addition of SDS-PAGE sample buffer 100 μ l).

10 Samples were heated for 5 min. at 100°C, and portions (10 to 15 μ l) were applied to a tris-glycine gel (4 to 20%) (Novex). After electrophoresis, proteins were electroblotted onto a polyvinylidenedifluoride (PVDF) membrane and then blocked with bovine serum albumin (5%) in 15 tris-buffered saline containing Tween-20 (0.05%) (TBST). Blots were probed with a monoclonal antibody (1:1000 dilution) to phosphotyrosine (Upstate Biotechnology) for a minimum of 1 hour at room temperature. Blots were washed with TBST, probed with an antibody to mouse immunoglobulin G 20 conjugated to alkaline phosphatase (Promega) (diluted 1:7500) for a minimum of 30 min. at room temperature. Reactive bands were visualized with 5-bromo-4-chloro-3-indoyl-1-phosphate and nitro-blue tetrazolium. Immunoblots were scanned with a Scan Jet Plus 25 (Hewlett-Packard) densitometer. Signal intensities for unstimulated MCF-7 cells were 20 to 30 units. Fully stimulated p185^{erbB2} yielded signals of 180 to 200 units. The 0.6 M NaCl pool, which contained most of the activity, was applied to a polyaspartic acid (PolyLC) column 30 equilibrated in 17 mM sodium phosphate (pH 6.8) containing ethanol (30%). A linear gradient from 0.3 M to 0.6 M NaCl in the equilibration buffer was used to elute bound proteins. A peak of activity (at ~0.45 M NaCl) was further

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fractionated on a C4 reversed-phase column (SynChropak RP-4) equilibrated in buffer containing TFA (0.1%) and acetonitrile (15%). Proteins were eluted from this column with an acetonitrile gradient from 25 to 40% over 60 min.

5 Fractions (1 ml) were collected, assayed for activity, and analyzed by SDS-PAGE on tris-glycine gels (4-20%, Novex). HPLC-purified HRG- α was digested with lysine C in SDS (0.1%), 10 mM dithiothreitol, 0.1 M NH₄HCO₃ (pH 8.0) for 20 hours at 37°C and the resultant fragments were resolved on a

10 Synchrom C4 column (4000A°, 0.2 by 10 cm). The column was equilibrated in 0.1% TFA and eluted with a 1-propanol gradient in 0.1% TFA (W. J. Henzel, J. T. Stults, C. Hsu, D. W. Aswad, *J. Biol. Chem.* **264**, 15905 (1989)). Peaks from the chromatographic run were dried under vacuum and sequenced.

15 One of the peptides (eluting at ~24% 1-propanol) gave the sequence [A]AEKEKTF[C]VNGGEXFMVKDLXNP (SEQ ID No. 162). Residues in brackets were uncertain and an X represents a cycle in which it was not possible to identify the amino acid. The initial yield was 8.5 pmol and the sequence did

20 not correspond to any known protein. Residues 1, 9, 15, and 22 were later identified in the cDNA sequence as cysteine. Direct sequencing of the ~45-kD band from a gel that had been overloaded and blotted onto a PVDF membrane revealed a low abundance sequence XEXKE[G][R]GK[G]K[G]KKKEXGXG[K] (SEQ

25 ID No. 163) with a very low initial yield (0.2 pmol). This corresponded to amino acid residues 2 to 22 of heregulin- α (Fig. 31), suggesting that serine 2 is the NH₂-terminus of proHRG- α . Although the NH₂ terminus was blocked, it was observed that occasionally a small amount of a normally

30 blocked protein may not be post-translationally modified. The NH₂ terminal assignment was confirmed by mass spectrometry of the protein after digestion with cyanogen bromide. The COOH-terminus of the isolated protein has not

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been definitely identified; however, by mixture sequencing of proteolytic digests, the mature sequence does not appear to extend past residue 241. Abbreviations for amino residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

As a source of cDNA clones, an oligo(dT)-primed λgt10 (T. V. Huynh, R. A. Young, R. W. Davis, λgt10 and λgt11 DNA Cloning Techniques: A Practical Approach, D. Glover, Ed. (IRG Press, Oxford, (1984)) cDNA library was constructed (U. Gubler and B. J. Hoffman, Gene 25, 263 (1983)) with mRNA purified (J. M. Chirwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, Biochemistry 18, 5294 (1979)) from MDA-MB-231 cells. The following eightfold degenerate antisense deoxyoligonucleotide encoding the 13-amino acid sequence AEKEKTFCVNGGE (SEQ ID No. 164) (13) was designed on the basis of human codon frequency optima (R. Lathe, J. Mol. Biol. 183, 1 (1985)) and chemically synthesized:

5'-CTCGCC (G OR T) CC (A OR G) TTCAC (A OR G)

20 CAGAAGGTCTTCCTTCTCAGC-3' (SEQ ID No. 165). For the purpose of probe design a cysteine was assigned to an unknown residue in the amino acid sequence. The probe was labeled by phosphorylation and hybridized under low-stringency conditions to the cDNA library. The proHRG- α protein was identified in this library. HRB-81 cDNA was identified by probing a second oligo(dT)-primed λgt10 library made from MDA-MB-231 cell mRNA with sequences derived from both the 5' and 3' ends of proHRG- α . Clone 13 (Fig. 2A) was a product of screening a primed

30 (5'-CCTCGCTCCTTCTTCTGCCCTTC-3' primer (SEQ ID No. 166); proHRG- α antisense nucleotides 33 to 56) MDA-MB-231 λgt10 library with 5' HRG- α sequence. A sequence corresponding to the 5' end of clone 13 as the probe was used to identify

proHRG82 and proHRG83 in a third oligo(dT)-primed λgt10 library derived from MDA-MB-231 cell mRNA. Two cDNA clones encoding each of the four HRGs were sequenced (F. Sanger, S. Milken, A. R. Coulson, Proc. Natl. Acad. Sci. U.S.A. 74, 5463 (1977)). Another cDNA designated clone 84 has an amino acid sequence identical to proHRG82 through amino acid 420. A stop codon at position 421 is followed by a different 3'-untranslated sequence.

EXAMPLE 9

10 Isolation of a Further Splicing Variant

The methods in Example 6 produced four closely related sequences (heregulin α, B1, B2, B3) which arise as a result of splicing variation. Peles et al. (Cell 69, 205 (1992)), and Wen et al. (Cell 69, 559 (1992)) have isolated another 15 splicing variant (from rat) using a similar purification and cloning approach to that described in Examples 1-4 and 6 involving a protein which binds to p185^{erbB2}. The cDNA clone was obtained as follows (via the purification and sequencing of a p185^{erbB2} binding protein from a transformed 20 rat fibroblast cell line).

A p185^{erbB2} binding protein was purified from conditioned medium as follows. Pooled conditioned medium from three harvests of 500 roller bottles (120 liters total) was cleared by filtration through 0.2 μ filters and concentrated 25 31-fold with a Pelicon ultrafiltration system using membranes with a 20kd molecular size cutoff. All the purification steps were performed by using a Pharmacia fast protein liquid chromatography system. The concentrated material was directly loaded on a column of 30 heparin-Sepharose (150 ml, preequilibrated with phosphate-buffered saline (PBS)). The column was washed

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with PBS containing 0.2 M NaCl until no absorbance at 280 nm wavelength could be detected. B und proteins were then eluted with a continuous gradient (250 ml) of NaCl (from 0.2 M to 1.0 M), and 5 ml fractions were collected. Samples 5 (0.01 ml of the collected fractions were used for the quantitative assay of the kinase stimulatory activity. Active fractions from three column runs (total volume = 360 ml) were pooled, concentrated to 25 ml by using a YM10 ultrafiltration membrane (Amicon, Danvers, MA), and ammonium 10 sulfate was added to reach a concentration of 1.7 M. After clearance by centrifugation (10,000 x g, 15 min.), the pooled material was loaded on a phenyl-Superose column (HR10/10, Pharmacia). The column was developed with a 45 ml gradient of $(\text{NH}_4)_2\text{SO}_4$ (from 1.7 M to no salt) in 0.1 M 15 Na_2PO_4 (pH 7.4), and 2 ml fractions were collected and assayed (0.002 ml per sample) for kinase stimulation (as described in Example 6). The major peak of activity was pooled and dialyzed against 50 mM sodium phosphate buffer (pH 7.3). A Mono-S cation-exchange column (HR5/5, 20 Pharmacia) was preequilibrated with 50 mM sodium phosphate. After loading the active material (0.884 mg of protein; 35 ml), the column was washed with the starting buffer and then developed at a rate of 1 ml/min. with a gradient of NaCl. The kinase stimulatory activity was recovered at 0.45-0.55 M 25 salt and was spread over four fractions of 2 ml each. These were pooled and loaded directly on a Cu^{+2} chelating columns (1.6 ml, HR2/5 chelating Superose, Pharmacia). Most of the proteins adsorbed to the resin, but they gradually eluted with a 30 ml linear gradient of ammonium chloride (0-1 M). 30 The activity eluted in a single peak of protein at the range of 0.05 to 0.2 M NH_4Cl . Samples from various steps of purification were analyzed by gel electrophoresis followed by silver staining using a kit fr m ICN (Costa Mesa, CA),

and their protein contents were determined with a Coomassie blue dye binding assay using a kit from Bio-Rad (Richmond, CA).

The p44 protein (10 µg) was reconstituted in 200 µl of 0.1 M ammonium bicarbonate buffer (pH 7.8). Digestion was conducted with L-1-tosyl-amide 2-phenylethyl chloromethyl ketone-treated trypsin (Serva) at 37°C for 18 hr. at an enzyme-to-substrate ratio of 1:10. The resulting peptide mixture was separated by reverse-phase HPLC and monitored at 215 nm using a Vydac C4 micro column (2.1 mm i.d. x 15 cm, 300 Å) and an HP 1090 liquid chromatographic system equipped with a diode-array detector and a workstation. The column was equilibrated with 0.1% trifluoroacetic acid (mobile phase A), and elution was effected with a linear gradient from 0%-55% mobile phase B (90% acetonitrile in 0.1% trifluoroacetic acid) over 70 min. The flow rate was 0.2 ml/min. and the column temperature was controlled at 25°C. One-third aliquots of the peptide peaks collected manually from the HPLC system were characterized by N-terminal sequence analysis by Edman degradation. The fraction eluted after 27.7 min. (T27.7) contained mixed amino acid sequences and was further rechromatographed after reduction as follows: A 70% aliquot of the peptide fraction was dried in vacuo and reconstituted in 100 µl of 0.2 M ammonium bicarbonate buffer (pH 7.8). DTT (final concentration 2 mM) was added to the solution, which was then incubated at 37°C for 30 min. The reduced peptide mixture was then separated by reverse-phase HPLC using a Vydac column (2.1 mm i.d. x 15 cm). Elution conditions and flow rate were identical to those described above. Amino acid sequence analysis of the peptide was performed with a Model 477 protein sequencer (Applied Biosystems, Inc., Foster City, CA) equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer and a

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Model 900 data analysis system (Hunkapiller et al. (1986) In Methods of Protein Microcharacterization, J.E. Shively, ed. (Clifton, New Jersey: Humana Press p. 223-247). The protein was loaded onto a trifluoroacetic acid-treated glass fiber disc precycled with polybrene and NaCl. The PTH-amino acid analysis was performed with a micro liquid chromatography system (Model 120) using dual syringe pumps and reverse-phase (C-18) narrow bore columns (Applied Biosystems, 2.1 mm x 250 mm).

5 RNA was isolated from Rat1-EJ cells by standard procedures (Maniatis et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York (1982) and poly (A)⁺ was selected using an mRNA Separator kit (Clontech Lab, Inc., Palo Alto, CA). cDNA was synthesized with the Superscript 10 kit (from BRL Life Technologies, Inc., Bethesda, MD).

15 Column-fractionated double-strand cDNA was ligated into an Sall- and NotI-digested pJT-2 plasmid vector, a derivative of the pCD-X vector (Okayama and Berg, Mol. Cell Biol. 3: 280 (1983)) and transformed into DH10B *E. coli* cells by 20 electroporation (Dower et al., Nucl. Acids Res. 16: 6127 (1988)). Approximately 5×10^5 primary transformants were screened with two oligonucleotide probes that were derived from the protein sequences of the N-terminus of NDF (residues 5-24) and the T40.4 tryptic peptide (residues 25 7-12). Their respective sequences were as follows (N indicates all 4 nt):

(1) 5'-ATA GGG AAG GGC GGG GGA AGG GTC NCC CTC NGC
A T
AGG GCC GGG CTT GCC TCT GGA GCC TCT-3'

30 (2) 5'-TTT ACA CAT ATA TTC NCC-3'
C G G C

(1: SEQ ID No. 167; 2: SEQ ID N . 168)

The synthetic oligonucleotides were end-labeled with [γ -³²P]ATP with T4 polynucleotide kinase and used to screen replicate sets of nitrocellulose filters. The hybridization
5 solution contained 6 x SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 2 x Denhardt's solution, 50 μ g/ml salmon sperm DNA, and 20% formamide (for probe 1) or no formamide (for probe 2). The filters were washed at either 50°C with 0.5 x SSC, 0.2% SDS, 2 mM EDTA (for probe 1) or at
10 37°C with 2 x SSC, 0.2% SDS, 2 mM EDTA (for probe 2). Autoradiography of the filters gave ten clones that hybridized with both probes. These clones were purified by replating and probe hybridization as described above. The cDNA clones were sequenced using an Applied Biosystems
15 373A automated DNA sequencer and Applied Biosystems Taq DyeDeoxy[®] Terminator cycle sequencing kits following the manufacturer's instructions. In some instances, sequences were obtained using [³⁵S]dATP (Amersham) and Sequenase[®] kits from U.S. Biochemicals following the manufacturer's
20 instructions. Both strands of the cDNA clone 44 were sequenced by using synthetic oligonucleotides as primers. The sequence of the most 5' 350 nt was determined in seven independent cDNA clones. The resultant clone demonstrated the pattern shown in figure 30 (NDF).

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EXAMPLE 10

Strategies for Detecting Other Possible Splicing Variants
Alignment of the deduced amino acid sequences of the cDNA clones and PCR products of the bovine, and the published human (Fig. 31) and rat sequences show a high level of
30 similarity, indicating that these sequences are derived from homologous genes within the three species. The variable

number of messenger RNA transcripts detectable at the cDNA/PCR product level is probably due to extensive tissue-specific splicing. The patterns obtained and shown in Figure 30 suggests that other splicing variants exist. A 5 list of probable splicing variants is indicated in Figure 37. Many of these variants can be obtained by coding segment specific probing of cDNA libraries derived from different tissues and by PCR experiments using primer pairs specific to particular coding segments. Alternatively, the 10 variants can be assembled from specific cDNA clones, PCR products or genomic DNA regions via cutting and splicing techniques known to one skilled in the art. For example, a rare restriction enzyme cutting site in a common coding segment (e.g., A), can be used to connect the FBA amino 15 terminus of GGF2BPP5 to carboxy terminal sequences of GGF2BPP1, GGF2BPP2, GGF2BPP3, or GGF2BPP4. If the presence or the absence of coding segment E and/or G provide benefit for contemplated and stated uses, then these coding segments can be included in expression constructs. These variant 20 sequences can be expressed in recombinant systems and the recombinant products can be assayed to determine their level of Schwann cell mitogenic activity as well as their ability to bind and activate the p185^{erbB2} receptor.

EXAMPLE 11

25 Identification of Functional Elements of GGF

The deduced structures of the family of GGF sequences indicate that the longest forms (as represented by GGF2BPP4) encode transmembrane proteins where the extracellular part contains a domain which resembles 30 epidermal growth factor (see Carpenter and Wahl in Peptide Growth Factors and Their Receptors I pp. 69-133, Springer-Verlag, NY 1991). The positions of the cystein

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r sidues in coding s gm nts C and C/D or C/D' p ptid sequenc ar conserved with respect to th analogous residues in the epidermal growth factor (EGF) peptide sequence (see Figure 35, SEQ ID Nos. 151-153). This
5 suggests that the extracellular domain functions as receptor recognition and biological activation sites. Several of the variant forms lack the H, K, and L coding segments and thus may be expressed as secreted, diffusible biologically active proteins. GGF DNA sequences encoding polypeptides which
10 encompass the EGF-like domain (EGFL) can have full biological activity for stimulating glial cell mitogenic activity.

Membrane bound versions of this protein may induce Schwann cell proliferation if expressed on the surface of
15 neurons during embryogenesis or during nerve regeneration (where the surfaces of neurons are intimately associated with the surfaces of proliferating Schwann cells).

Secreted (non membrane bound) GGFs may act as classically diffusible factors which can interact with
20 Schwann cells at some distance from their point of secretion. Other forms may be released from intracells by sources via tissue injury and cell disruption. An example of a secreted GGF is the protein encoded by GGF2HBS5 (see example 6); this is the only GGF known which has been found
25 to be directed to the exterior of the cell (example 7). Secretion is probably mediated via an N-terminal hydrophobic sequence found only in region E, which is the N-terminal domain contained within recombinant GGF-II encoded by GGF2HBS5.

30 Other GGF's appear to be non-secreted (see example 6). These GGFs may be injury response forms which are released as a consequence of tissue damage.

Other regions of the predicted protein structure of GGF-II (encoded by GGF2HBS5) and other proteins containing regions B and A exhibit similarities to the human basement membrane heparin sulfate proteoglycan core protein (Kallunki,

- 5 P. and Tryggvason, K., Cell Biology Vol. 116, p. 559-571
(1992)). The peptide ADSGEY, which is located next to the second cysteine of the C2 immunoglobulin fold in these GGF's, occurs in nine of twenty-two C-2 repeats found in that basal lamina protein. This evidence strongly suggests
10 that these proteins may associate with matrix proteins such as those associated with neurons and glia, and may suggest a method for sequestration of glial growth factors at target sites.

EXAMPLE 12

15 Purification of GGFs from Recombinant Cells

In order to obtain full length or portions of GGFs to assay for biological activity, the proteins can be overproduced using cloned DNA. Several approaches can be used. A recombinant *E. coli* cell containing the sequences described above can be constructed. Expression systems such as pNH8a or pHH16a (Stratagene, Inc.) can be used for this purpose by following manufacturers procedures.
20 Alternatively, these sequences can be inserted in a mammalian expression vector and an overproducing cell line can be constructed. As an example, for this purpose DNA encoding a GGF, clone GGF2BPP5 has been expressed in both COS cells and Chinese hamster ovary cells (see Example 7) (J. Biol. Chem. 263, 3521-3527, (1981)). This vector containing GGF DNA sequences can be transfected into host
25 cells using established procedures.
30

Transient expression can be examined or G418-resistant clones can be grown in the presence of

methotrexate to select for cells that amplify the dhfr gene (contained on the pMSXND vector) and, in the process, co-amplify the adjacent GGF protein encoding sequence.

Because CHO cells can be maintained in a totally serum-free, 5 protein-free medium (Hamilton and Ham, *In Vitro* 13, 537-547 (1977)), the desired protein can be purified from the medium. Western analysis using the antisera produced in Example 9 can be used to detect the presence of the desired protein in the conditioned medium of the overproducing 10 cells.

The desired protein (rGGF-II) was purified from the medium conditioned by transiently expressing COS cells as follows. rGGF-II was harvested from the conditioned medium and partially purified using Cation Exchange Chromatography 15 (POROS-HS). The column was equilibrated with 33.3 mM MES at pH 6.0. Conditioned media was loaded at flow rate of 10 ml/min. The peak containing Schwann cell proliferation activity and immunoreactive (using the polyclonal antisera was against a GGFII peptide described above) was eluted with 20 50 mM Tris, 1M NaCl pH 8.0. (Figure 50A and 50B respectively).

rGGF-II is also expressed using a stable Chinese Hamster Ovary cell line. rGGF-II from the harvested conditioned media was partially purified using Cation 25 Exchange Chromatograph (POROS-HS). The column was equilibrated with PBS pH 7.4. Conditioned media was loaded at 10 ml/min. The peak containing the Schwann Cell Proliferative activity and immunoreactivity (using GGFII polyclonal antisera) was eluted with 50 mM Hepes, 500 mM 30 NaCl pH 8.0. An additional peak was observed at 50 mM Hepes, 1M NaCl pH 8.0 with both proliferation as well as immunoreactivity (Fig. 51).

POWERS THERAPY

rGGF-II can be further purified using Hydrophobic Interaction Chromatography as a high resolution step; Cation Exchange/Reverse phase Chromatography (if needed as second high resolution step); a viral inactivation step and a DNA 5 removal step such as Anion Exchange chromatography.

Detailed description of procedures used are as follows:

Schwann Cell Proliferation Activity of the recombinant GGF-II peak eluted from the Cation Exchange 10 column was determined as follows: Mitogenic responses of the cultured Schwann cells were measured in the presence of 5 μ M forskolin using the peak eluted by 50 mM Tris 1 M NaCl pH 8.0. The peak was added at 20 l, 10 l (1:10) 10 l and (1:100) 10 l. Incorporation of 125 I-Uridine was determined 15 and expressed as (CPM) following an 18-24 hour exposure.

An immunoblot using polyclonal antibody raised against a peptide of GGF-II was carried out as follows: 10 μ l of different fractions were run on 4-12% gradient gels. The gels were transferred on to Nitrocellulose paper, and 20 the nitrocellulose blots were blocked with 5% BSA and probed with GGF-II-specific antibody (1:250 dilution). 125 I protein A (1:500 dilution, Specific Activity = 9.0/ci/g) was used as the secondary antibody. The immunoblots were exposed to Kodax X-Ray films for 6 hours. The peak 25 fractions eluted with 1 M NaCl showed a broad immunoreactive band at 65-90 Kd which is the expected size range for GGFII and higher molecular weight glycoforms.

GGF-II purification on cation exchange columns was performed as follows: CHO cell conditioned media expressing 30 rGGFII was loaded on the cation exchange column at 10 ml/min. The column was equilibrated with PBS pH 7.4. The

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elution was achieved with 50 mM Hepes 500 mM NaCl pH 8.0 and 50 mM Hepes 1M NaCl pH 8.0 respectively. All fractions were analyzed using the Schwann cell proliferation assay (CPM) described herein. The protein concentration (mg/ml) was

5 determined by the Bradford assay using BSA as the standard.

A Western blot using 10 µl of each fraction was performed. As indicated in Figure 51A and 51B, immunoreactivity and the Schwann cell activity co-migrates.

The Schwann cell mitogenic assay described herein
10 may be used to assay the expressed product of the full length clone or any biologically active portions thereof. The full length clone GGF2BPP5 has been expressed transiently in COS cells. Intracellular extracts of transfected COS cells show biological activity when assayed
15 in the Schwann cell proliferation assay described in Example 1. In addition, the full length clone encoding GGF2HBS5 has been expressed stably in CHO and insect viral systems (Example 7) cells. In this case both cell extract and conditioned media show biological activity in the Schwann
20 cell proliferation assay described in Example 1. Any member of the family of splicing variant complementary DNA's derived from the GGF gene (including the Heregulins) can be expressed in this manner and assayed in the Schwann cell proliferation assay by one skilled in the art.

25 Alternatively, recombinant material may be isolated from other variants according to Wen et al. (Cell 69, 559 (1992)) who expressed the splicing variant Neu differentiation factor (NDF) in COS-7 cells. cDNA clones inserted in the pJT-2 eukaryotic plasmid vector are under
30 the control of the SV40 early promoter, and are 3'-flanked with the SV40 termination and polyadenylation signals. COS-7 cells were transfected with the pJT-2 plasmid DNA by electroporation as follows: 6 x 10⁶ cells (in 0.8 ml of

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DMEM and 10% FBS) were transferred to a 0.4 cm cuvett and mixed with 20 µg of plasmid DNA in 10 µl of TE solution (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). Electroporation was performed at room temperature at 1600 V and 25 µF using a
5 Bio-Rad Gene Pulser apparatus with the pulse controller unit set at 200 ohms. The cells were then diluted into 20 ml of DMEM, 10% FBS and transferred into a T75 flask (Falcon). After 14 hr. of incubation at 37°C, the medium was replaced with DMEM, 1% FBS, and the incubation continued for an
10 additional 48 hr. Conditioned medium containing recombinant protein which was harvested from the cells demonstrated biological activity in a cell line expressing the receptor for this protein. This cell line (cultured human breast carcinoma cell line AU 565) was treated with recombinant
15 material. The treated cells exhibited a morphology change which is characteristic of the activation of the erbB2 receptor. Conditioned medium of this type also can be tested in the Schwann cell proliferation assay.

EXAMPLE 13

20 Purification and Assay of Other Proteins which bind p185^{erbB2} Receptor

I. Purification of gp30 and p70

Lupu et al. (Science 249, 1552 (1990)) and Lippman and Lupu (patent application number PCT/US91/03443 (1990)),
25 hereby incorporated by reference, have purified a protein from conditioned media of a human breast cancer cell line MDA-MB-231, as follows.

Conditioned media collections were carried using well-known procedures. The media was concentrated 100-fold
30 in an Amicon ultra-filtration cell (YM5 membrane) (Amicon, Danvers, MA). Once clarified and concentrated, the media were stored at -20°C while consecutive collections were made

during the following days. The concentrated medium was dialyzed using Spectra/pore[®] 3 tubing (Spectrum Medical Industries, Los Angeles, CA) against 100 volumes of 0.1 M acetic acid over a two day period at 4°C. The material that 5 precipitated during dialysis was removed by centrifugation at 4000 rpm for 30 min. at 4°C; protease inhibitors were added. The clarified sample was then lyophilized.

Lyophilized conditioned medium was dissolved in 1 M acetic acid to a final concentration of about 25 mg/ml total 10 protein. Insoluble material was removed by centrifugation at 10,000 rpm for 15 minutes. The sample was then loaded onto a Sephadex G-100 column (XK 16, Pharmacia, Piscataway, NJ), was equilibrated and was subjected to elution with 1 M acetic acid at 4°C with an upward flow of 30 ml/hr. 100 ng 15 of protein was processed from 4 ml of 100-fold concentrated medium. Fractions containing 3 ml of eluate were lyophilized and resuspended in 300 µl PBS for assay and served as a source for further purification.

Sephadex G-100 purified material was run on 20 reversed-phase high pressure liquid chromatography (HPLC). The first step involved a steep acetonitrile gradient. Steep acetonitrile gradient and all other HPLC steps were carried out at room temperature after equilibration of the C3-Reversed phase column with 0.05% TFA (Trifluoroacetic 25 acid) in water (HPLC-grade). The samples were loaded and fractions were eluted with a linear gradient (0-45% acetonitrile in 0.05% TFA) at a flow rate of 1 ml/min. over a 30 minute period. Absorbance was monitored at 280 nm. One ml fractions were collected and lyophilized before 30 analysis for EGF receptor-competing activity.

A second HPLC step involved a shallow acetonitrile gradient. The pool of active fractions from the previous HPLC step was rechromatographed over the same column.

Elution was performed with a 0-18% acetonitrile gradient in 0.05% TFA over a 5 minute period followed by a linear 18-45% acetonitrile gradient in 0.05% TFA over a 30 minute period. The flow rate was 1.0 ml/min. and 1 ml fractions were collected. Human TGF α -like factor was eluted at a 30-32% acetonitrile concentration as a single peak detectable by RRA.

Lupu et al. (Proc. Natl. Acad. Sci. 89, 2287 (1992)) purified another protein which binds to the p185^{erbB2} receptor. This particular protein, p75, was purified from conditioned medium used for the growth of SKBr-3 (a human breast cancer cell line) propagated in improved Eagle's medium (IMEM: GIBCO) supplemented with 10% fetal bovine serum (GIBCO). Protein p75 was purified from concentrated (100X) conditioned medium using a p185^{erbB2} affinity column. The 94 Kilodalton extracellular domain of p185^{erbB2} (which binds p75) was produced via recombinant expression and was coupled to a polyacrylamide hydrazido-Sepharose affinity chromatography matrix. Following coupling the matrix was washed extensively with ice cold 1.0 M HCl and the beads were activated with 0.5 M NaNO₂. The temperature was maintained at 0°C for 20 minutes and this was followed by filtration and washing with ice cold 0.1 M HCl. 500 ml of concentrated conditioned medium was run through the beads by gravity. The column was washed and eluted stepwise with 1.0 M citric acid at pH values from 4.0 to 2.0 (to allow dissociation of the erbB2 and p75). All fractions were desalted on Pharmacia PD10 columns. Purification yielded a homogeneous polypeptide of 75kDa at 3.0-3.5 elution pH (confirmed by analysis on SDS/PAGE by silver staining).

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II. Binding of gp30 to p185^{erbB2}

The purified gp30 protein was tested in an assay to determine if it bound to p185^{erbB2}. A competition assay with a monoclonal antibody against p185^{erbB2}. The gp30 5 protein displaced antibody binding to p185^{erbB2} in SK-BR-3 and MDA-MB-453 cells (human breast carcinoma cell lines expressing the p185^{erbB2} receptor). Schwann cell proliferation activity of gp30 can also be demonstrated by treating Schwann cell cultures with purified gp30 using the 10 assay procedure described in Examples 1-3.

III. Binding of p75 to p185^{erbB2}

To assess whether the 75-kDa polypeptide (p75) obtained from SKBr-3 conditioned medium was indeed a ligand for the erbB2 oncoprotein in SKBr-3 cells, a competition 15 assay as described above for gp30 was used. It was found that the p75 exhibited binding activity, whereas material from other chromatography fractions did not show such activity (data not shown). The flow-through material showed some binding activity. This might be due to the presence of 20 shed erbB2 ECD.

IV. Other p185^{erbB2} ligands

Pelles et al. (Cell 69, 205 (1992)) have also purified a 185^{erbB2} stimulating ligand from rat cells, (NDF, see Example 8 for method). Holmes et al. (Science 256, 1205 25 (1992)) have purified Heregulin α from human cells which binds and stimulates 185^{erbB2} (see example 6). Tarakovsky et al. Oncogene 6:218 (1991) have demonstrated bending of a 25 kD polypeptide isolated from activated macrophages to the Neu receptor, a p185^{erbB2} homology, herein incorporated by 30 reference.

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VI. NDF Isolation

Yarden and Peles (Biochemistry 30, 3543 (1991)) have identified a 35 kilodalton glycoprotein which will stimulate the $185^{\text{erbB}2}$ receptor. The protein was identified in 5 conditioned medium according to the following procedure. Rat I-EJ cells were grown to confluence in 175- cm^2 flasks (Falcon). Monolayers were washed with PBS and left in serum-free medium for 10-16 h. The medium was discarded and replaced by fresh serum-free medium that was collected after 10 3 days in culture. The conditioned medium was cleared by low-speed centrifugation and concentrated 100-fold in an Amicon ultrafiltration cell with a YM2 membrane (molecular weight cutoff of 2000). Biochemical analyses of the neu stimulatory activity in conditioned medium indicate that the 15 ligand is a 35-kD glycoprotein that it is heat stable but sensitive to reduction. The factor is precipitable by either high salt concentrations or acidic alcohol. Partial purification of the molecule by selective precipitation, heparin-agarose chromatography, and gel filtration in dilute 20 acid resulted in an active ligand, which is capable of stimulating the protooncogenic receptor but is ineffective on the oncogenic neu protein, which is constitutively active. The purified fraction, however, retained the ability to stimulate also the related receptor for EGF, 25 suggesting that these two receptors are functionally coupled through a bidirectional mechanism. Alternatively, the presumed ligand interacts simultaneously with both receptors. The presented biochemical characteristic of the factor may be used to enable a completely purified factor 30 with which to explore these possibilities.

In other publications, Davis et al. (Biochem. Biophys. Res. Commun. 179, 1536 (1991), Proc. Natl. Acad. Sci. 88, 8582 (1991) and Greene et al., PCT patent

application PCT/US91/02331 (1990)) describe the purification of a protein from conditioned medium of a human T-cell (ATL-2) cell line.

ATL-2 cell line is an IL-2-independent HTLV-1 (+) T
5 cell line. Mycoplasm-free ATL-2 cells were maintained in RPMI 1640 medium containing 10% FCB as the culture medium (10% FCS-RPMI 1640) at 37°C in a humidified atmosphere with 5% CO₂.

For purification of the proteinaceous substance,
10 ATL-2 cells were washed twice in 1 x PBS and cultured at 3 x 10⁵ ml in serum-free RPMI 1640 medium/2 mM L-glutamine for seventy-two hours followed by pelleting of the cells. The culture supernatant so produced is termed "conditioned medium" (C.M.).

C.M. was concentrated 100 fold, from 1 liter to 10 ml, using a YM-2 Diaflo membrane (Amicon, Boston, MA) with a 1000d cutoff. For use in some assays, concentrated C.M. containing components greater than 1000 MW were rediluted to original volume with RPMI medium. Gel electrophoresis using
20 a polyacrylamide gradient gel (Integrated Separation Systems, Hyde Park, MD or Phorecast System by Amersham, Arlington Heights, IL) followed by silver staining of some of this two column purified material from the one liter preparation revealed at least four to five bands of which
25 the 10kD and 20kD bands were unique to this material. Passed C.M. containing components less than 1000 MW were used without dilution.

Concentrated conditioned medium was filter sterilized with a .45μ uniflo filter (Schleicher and Schuell, Keene, NH) and then further purified by application to a DEAE-SW anion exchange column (Waters, Inc., Milford, MA) which had been preequilibrated with 10mM Tris-Cl, pH 8.1 Concentrated C.M. proteins representing one liter of

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original ATL-2 condition d medium p r HPLC run wer absorbed to the column and then eluted with a linear gradient of 0mM to 40mM NaCl at a flow rate of 4 ml/min. Fractions were assayed using an in vitro immune complex kinase assay with
5 10% of the appropriate DEAE fraction (1 column purified material) or 1% of the appropriate C18 fractions (two column purified material). The activity which increased the tyrosine kinase activity of p185c-neu in a dose-dependent manner using the in vitro immune complex kinase assay was
10 eluted as one dominant peak across 4 to 5 fractions (36-40) around 220 to 240 mM of NaCl. After HPLC-DEAE purification, the proteins in the active fractions were concentrated and pooled, concentrated and subjected to C18 (million matrix) reverse phase chromatography (Waters, Inc., Milford, MA)
15 (referred to as the C18+1 step or two column purified material). Elution was performed under a linear gradient of 2-propanol against 0.1% TFA. All the fractions were dialyzed against RPMI 1640 medium to remove the 2-propanol and assayed using the in vitro immune complex kinase assay,
20 described below, and a 1% concentration of the appropriate fraction. The activity increasing the tyrosine kinase activity of p185c-neu was eluted in two peaks. One eluted in fraction 11-13, while a second, slightly less active peak of activity eluted in fractions 20-23. These two peaks
25 correspond to around 5 to 7% of isopropanol and 11 to 14% isopropanol respectively. C18#1 generated fractions 11-13 were used in the characterization studies. Active fractions obtained from the second chromatographic step were pooled, and designated as the proteinaceous substance sample.

30 A twenty liter preparation employed the same purification strategy. The DEAE active fractions 35-41 were pooled and subjected to c18 chromatography as discussed above. C18#1 fractions 11-13 and 21-24 both had

dose-dependent activity. The pool of fractions 11-13 was subjected to an additional C18 chromatographic step (referred to as C18#2 or three column purified material). Again, fractions 11-13 and 21-24 had activity. The dose response of fraction 23 as determined by *in vitro* immune complex kinase assay as described in Example 8 may be obtained upon addition of 0.005% by volume fraction 23 and 0.05% by volume fraction 23. This represents the greatest purity achieved.

Molecular weight ranges were determined based on gel filtration chromatography and ultrafiltration membrane analysis. Near equal amounts of tyrosine kinase activity were retained and passed by a 10,000 molecular weight cut off filter. Almost all activity was passed by a 30,000 molecular weight cut off filter. Molecular weight ranges for active chromatographic fractions were determined by comparing fractions containing dose-dependent neu-activating activity to the elution profiles of a set of protein molecular weight standards (Sigma Chemical Co., St. Louis, MO) generated using the same running conditions. A low molecular weight region of activity was identified between 7,000 and 14,000 daltons. A second range of activity ranged from about 14,000 to about 24,000 daltons.

After gel electrophoresis using a polyacrylamide gradient gel (Integrated Separation Systems, Hyde Park, MD or Phorecase System by Amersham, Arlington Heights, IL), silver staining of the three-column purified material (c18#2) was done with a commercially available silver staining kit (BioRad, Rockville Centre, NY). Fraction 21, 22, 23, and 24 from c18#2 purification of the twenty liter preparation were run with markers. Fractions 22 and 23 showed the most potent dose response in the $^{185}\text{erbB}2$ (neu) kinase assay (see below). The fact that selected molecular

weight fractions interact with 185^{erbB2} was demonstrated with an immune complex kinase assay.

Huang et al. (1992, J. Biol. Chem. 257:11508-11512), hereby incorporated by reference, have isolated an additional neu/erb B2 ligand growth factor from bovine kidney. The 25 kD polypeptide factor was isolated by a procedure of column fractionation, followed by sequential column chromatography on DEAE/cellulose (DE52), Sulfadex (sulfated Sephadex G-50), heparin-Sepharose 4B, and Superdex 75 (fast protein liquid chromatography). The factor, NEL-GF, stimulates tyrosine-specific autophosphorylation of the neu/erb B2 gene product.

VII. Immune complex assay NDF for ligand binding to p185^{erbB2}: This assay reflects the differences in the autophosphorylation activity of immunoprecipitated p185 driven by pre-incubation of PN-NR6 cell lysate with varying amounts of ATL-2 conditioned medium (C.H.) or proteinaceous substance and is referred to hereinafter as neu-activating activity.

Cell lines used in the immune complex kinase assay were obtained, prepared and cultured according to the methods disclosed in Kokai et al., Cell 55, 287-292 (July 28, 1989) the disclosures of which are hereby incorporated by reference as if fully set forth herein, and U.S. application serial number 386,820 filed July 27, 1989 in the name of Mark I. Green entitled "Methods of Treating Cancerous Cells with Anti-Receptor Antibodies", the disclosures of which are hereby incorporated by reference as if fully set forth herein.

Cell lines were all maintained in DMEM medium containing 5% FCS as the culture medium (5% FCS-DMEM) at 37°C in a humidified atmosphere with 5% CO₂.

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Dense cultures of cells in 150 mm dishes were washed twice with cold PBS, scraped into 10 ml of freeze-thaw buffer (150 mM NaCl, 1 mM MgCl₂, 20 mM Hepes, pH 7.2, 10% Glycerol, 1 mM EDTA, 1% Aprotinin), and centrifuged (600 x g, 10 minutes). Cell pellets were resuspended in 1 ml Lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 3% Brij 35, 1 mM EDTA, 1.5 mM MgCl₂, 1% Aprotinin, 1 mM EGTA, 20 μM Na₃VO₄, 10% Glycerol) and rotated for thirty minutes at 4°C. All chemicals were from Sigma Chemical Co., St. Louis, Mo, unless otherwise indicated. The insoluble materials were removed by centrifugation at 40,000 x g for thirty minutes. The clear supernatant which was subsequently used is designated as cell lysate.

The cell lysates were incubated for fifteen minutes with 50 μl of 50% (volume/volume) Protein A-sepharose (Sigma Chemical Co., St. Louis, Missouri), and centrifugated for two minutes to preclear the lysates. 50 μl aliquots of precleared cell lysate were incubated on ice for fifteen minutes with conditioned medium, proteinaceous substance, or other factors as specified, in a final volume of 1 ml with lysis buffer. The sample was then incubated with 5 μg of 7.16.4 monoclonal antibody, which recognizes the extracellular domain of the p185neu and p185c-neu, or other appropriate antibodies, for twenty minutes on ice, followed by a twenty minute incubation with 50 μl of 50% (vol/vol) protein A-Sepharose with rotation at 4°C. Immune complexes were collected by centrifugation, washed four times with 500 μl of washing buffer (50 mM Hepes, pH 7.5, 0.1%, Brij 35, 150 mM NaCl, 2 mM EDTA, 1% Aprotinin, 30 μM Na₃VO₄), then twice with reaction buffer (20 mM Hepes (pH 7.4), 3 mM MnCl₂ and 0.1% Brij 35, 30 μM Na₃VO₄). Pellets were resuspended in 50 μl of reaction buffer and (Gamma-³²P)-ATP (Amersham, Arlington Heights, IL) was added giving a final

concentration of 0.2 µm. The samples were incubated at 27°C for twenty minutes or at 4°C for 25 minutes with purer samples. The reactions were terminated by addition of 3 x SDS sample buffer containing 2 mM ATP and 2 mM EDTA and then 5 incubating them at 100°C for five minutes. The samples were then subjected to SDS-PAGE analysis on 10% acrylamide gels. Gels were stained, dried, and exposed to Kodak XAR or XRP film with intensifying screens.

VIII. Purification of acetylcholine receptor inducing 10 activity (ARIA)

ARIA, a 42 kD protein which stimulates acetylcholine receptor synthesis, has been isolated in the laboratory of Gerald Fischbach (Falls et al., Cell 72:801-815 (1993)). ARIA induces tyrosine phosphorylation of a 185 KDa muscle 15 transmembrane protein which resembles p185^{erbB2}, and stimulates acetylcholine receptor synthesis in cultured embryonic myotubes. Sequence analysis of cDNA clones which encode ARIA shows that ARIA is a member of the GGF/erbB2 ligand group of proteins, and this is potentially useful in 20 the glial cell mitogenesis stimulation and other applications of, e.g., GGF2 described herein.

EXAMPLE 14

Protein tyrosine phosphorylation mediated by GGF in Schwann cells

25 Rat Schwann cells, following treatment with sufficient levels of Glial Growth Factor to induce proliferation, show stimulation of protein tyrosine phosphorylation (figure 36). Varying amounts of partially purified GGF were applied to a primary culture of rat 30 Schwann cells according to the procedure outlined in Example 3. Schwann cells were grown in DMEM/10% fetal calf serum/5

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μM forskolin/ $0.5\mu\text{g}$ per mL GGF-CM (0.5mL per well) in poly D-lysine coated 24 well plates. When confluent, the cells were fed with DMEM/10% fetal calf serum at 0.5mL per well and left in the incubator overnight to quiesce. The 5 following day, the cells were fed with 0.2mL of DMEM/10% fetal calf serum and left in the incubator for 1 hour. Test samples were then added directly to the medium at different concentrations and for different lengths of time as required. The cells were then lysed in boiling lysis buffer 10 (sodium phosphate, 5mM , pH 6.8; SDS, 2%; β -mercaptoethanol, 5%; dithiothreitol, 0.1M; glycerol, 10%; Bromophenol Blue, 0.4% ; sodium vanadate, 10mM), incubated in a boiling water bath for 10 minutes and then either analyzed directly or frozen at -70°C . Samples were analyzed by running on 7.5% 15 SDS-PAGE gels and then electroblotting onto nitrocellulose using standard procedures as described by Towbin et al. (1979) Proc. Natl. Acad. Sci. USA 76:4350-4354. The blotted nitrocellulose was probed with antiphosphotyrosine antibodies using standard methods as described in Kamps and 20 Selton (1988) Oncogene 2:305-315. The probed blots were exposed to autoradiography film overnight and developed using a standard laboratory processor. Densitometric measurements were carried out using an Ultrascan XL enhanced laser densitometer (LKB). Molecular weight assignments were 25 made relative to prestained high molecular weight standards (Sigma). The dose responses of protein phosphorylation and Schwann cell proliferation are very similar (figure 36). The molecular weight of the phosphorylated band is very close to the molecular weight of p₁₈₅^{erbB2}. Similar results 30 were obtained when Schwann cells were treated with conditioned media prepared from COS cells transfected with the GGF2HB5 clone. These results correlate well with the

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expected interaction of the GGFs with and activation of 185^{erbB2}.

This experiment has been repeated with recombinant GGF-II. Conditioned medium derived from a CHO cell line 5 stably transformed with the GGF-II clone (GGF2HBS5) stimulates protein tyrosine phosphorylation using the assay described above. Mock transfected CHO cells fail to stimulate this activity (Fig. 52).

EXAMPLE 15

10 Assay for Schwann cell Proliferation by Protein Factor from the MDA-MB-231 cell line.

Schwann cell proliferation is mediated by conditioned medium derived from the human breast cancer cell line MDA-MB-231. On day 1 of the assay, 10⁴ primary rat 15 Schwann cells were plated in 100 µl of Dulbecco's Modified Eagle's medium supplemented with 5% fetal bovine plasma per well in a 96 well microtiter plate. On day 2 of the assay, 10 µl of conditioned medium (from the human breast cancer cell line MDA-MB-231, cultured as described in Example 6) 20 was added to each well of the microtiter plate. One day 6, the number of Schwann cells per plate was determined using an acid phosphatase assay (according to the procedure of Connolly et al. Anal. Biochem. 152: 136 (1986)). The plate was washed with 100 µl of phosphate buffered saline (PBS) 25 and 100 µl of reaction buffer (0.1M sodium acetate, (pH 5.5)), 0.1% Triton X-100, and 10 mM p-nitrophenyl phosphate) was added per well. The plate was incubated at 37°C for two hours and the reaction was stopped by the addition of 10 µl of 1N NaOH. The optical density of each sample was read in 30 a spectrophotometer at 410 nm. A 38% stimulation of cell number over Schwann cells treated with conditioned medium

from a control cell line (HS-294T, a non-producer of erbB-2 ligand) was observed. This result shows that a protein secreted by the MDA-MB-231 cell line (which secretes a p185^{erbB2} binding activity) stimulates Schwann cell proliferation.

EXAMPLE 16

N-glycosylation of GGF

The protein sequence predicted from the cDNA sequence of GGF-II candidate clones GGF2BPP1,2 and 3 contains a number of consensus N-glycosylation motifs. A gap in the GGFII02 peptide sequence coincides with the asparagine residue in one of these motifs, indicating that carbohydrate is probably bound at this site.

N-glycosylation of the GGFs was studied by observing mobility changes on SDS-PAGE after incubation with N-glycanase, an enzyme that cleaves the covalent linkages between carbohydrate and asparagine residues in proteins.

N-Glycanase treatment of GGF-II yielded a major band of MW 40-42 kDa and a minor band at 45-48 kDa. Activity elution experiments under non-reducing conditions showed a single active deglycosylated species at ca 45-50 kDa.

Activity elution experiments with GGF-I also demonstrate an increase in electrophoretic mobility when treated with N-Glycanase, giving an active species of MW 26-28 kDa. Silver staining confirmed that there is a mobility shift, although no N-deglycosylated band could be assigned because of background staining in the sample used.

Deposit

Nucleic acid encoding GGF-II (cDNA, GGF2HBS5) protein (Example 6) in a plasmid pBluescript 5k, under the

control of the T7 promoter, was deposited in the American Type Culture Collection, Rockville, Maryland, on September 2, 1992, and given ATCC Accession No. 75298. Applicant acknowledges its responsibility to replace this plasmid

- 5 should it become non-viable before the end of the term of a patent issued hereon, and its responsibility to notify the ATCC of the issuance of such a patent, at which time the deposit will be made available to the public. Prior to that time the deposit will be made available to the Commissioner
10 of Patents under the terms of 37 CFR §1.14 and 35 USC §112.

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